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<b>(54) Title:</b> MANUAL <i>IN SITU</i> HYBRIDIZATION ASSAY  <b>(57) Abstract</b> <p>A rapid, sensitive <i>in situ</i> hybridization assay is provided which will detect as few as 1-5 copies of target biopolymer per cell and may be accomplished in 2-4 hours. There is provided a quantitative assay which may be used to diagnose and monitor treatment of diseases.</p>		

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-1-

MANUAL IN SITU HYBRIDIZATION ASSAY

## BACKGROUND OF THE INVENTION

1. Field of the invention.

The present invention relates to the field of in situ hybridization assays useful for detecting as few as 1-5 copies of target nucleic acid per cell. This assay method significantly increases the sensitivity of detection of nucleic acids over other known methods. In addition, this hybridization method is accomplished with far greater speed than has been reported for other in situ assays. This present invention also provides a method for the rapid and sensitive detection of nuclear acids and proteins in the same cell.

2. Description of the prior art.

In situ hybridization provides a technique for the determination and quantitation of biopolymers such as nucleic acids (DNA and RNA) and proteins in tissues at the single cell level. Such hybridization techniques can detect the presence or absence of specific genes in tissues at the single cell level. In situ hybridization procedures may also be utilized to detect the expression

1 of gene products at the single cell level.

By the use of specific nucleic acid (RNA or DNA) probes, genetic markers for infection and other disease states may be detected. Certain genetic diseases are characterized by the presence of genes which are not  
5 present in normal tissue. Other diseased conditions are characterized by the expression of RNAs or RNA translation products (i.e. peptides or proteins) which are not expressed in normal cells. Some disease states are  
10 characterized by the absence of certain genes or gene portions, or the absence or alteration of expression of gene products or proteins.

Current methods allow the detection of these markers but are relatively time consuming and of limited  
15 sensitivity. Hybridization techniques are based on the ability of single stranded DNA or RNA to pair (or hybridize) with a complementary nucleic acid strand. This hybridization reaction allows the development of specific probes that can identify the presence of specific genes  
(DNA), or polynucleotide sequences or the transcription  
20 and expression of those genes (mRNA).

Solution hybridization methods which require the destruction of the cell and the isolation of the nucleic acids from the cell prior to carrying out the  
25 hybridization reaction sacrifice the cellular integrity, spatial resolution and sensitivity of detection. In situ hybridization allows the detection of RNA or DNA sequences within individual cells. In situ hybridization yields greater sensitivity than solution hybridization by means  
30 of eliminating the dilution of a particular target gene, nucleic acid, or protein by the surrounding and extraneous RNA and DNA of other cells. In situ hybridization also allows for the simultaneous detection of multiple substances, i.e. genes, nucleic acids or proteins within  
35 individual cells, permitting the identification of a

1 particular cell expressing a cellular gene or viral  
sequence. In addition, since in situ hybridization  
analysis is performed and quantitated for single cells,  
minimal sample and reagents are required.

5 Prior to the present invention, in situ  
hybridization procedures were only capable of detecting  
nucleic acids present at greater than ten copies per  
cell. Such procedures required at least 8 hrs. to over 14  
days to perform. Prior in situ procedures were neither  
10 quantitative nor capable of performing multiple  
simultaneous detections.

#### SUMMARY OF THE INVENTION

It is an object of the present invention to  
15 provide an in situ hybridization procedure capable of  
detecting polynucleotides when present at a concentration  
as low as 1-5 copies per cell.

It is a further object of the present invention  
to provide an in situ hybridization procedure capable of  
20 detecting more than one target molecule in an individual  
cell.

It is a further object of the present invention  
to provide an in situ hybridization procedure that could  
be carried out within about two to four hours.

25 It is a further object of the present invention  
to provide an in situ hybridization procedure that could  
be quantitative for as few as 1-5 molecules of target  
nucleic acid per cell.

It is a further object of the present invention  
30 to provide an in situ hybridization procedure that could  
simultaneously detect multiple biopolymers.

The present invention provides a method for the  
detection of biopolymers within individual cells or tissue  
sections deposited on a solid support. Optimization of  
35 each step of the procedure as provided by the present

1 invention allows a rapid, sensitive hybridization assay.  
Target biopolymer molecules may be quantitated at a level  
of as few as 1-5 molecules per cell. This hybridization  
assay may be used to detect levels of polynucleotides in  
5 cells such as bone marrow and peripheral blood, in tissue  
sections or in tissue cultured cells. The hybridization  
procedure of the present invention can detect  
polynucleotides in single cells with the sensitivity of as  
few as 1-5 molecules per cell in as little as 2-4 hours.  
10 This procedure also allows for the simultaneous detection  
of more than one different polynucleotide sequence in an  
individual cell. The present invention also allows  
detection of proteins and polynucleotides in the same  
cell.

15 Briefly, cells, either as single cell suspensions  
or as tissue slices were deposited on solid supports such  
as glass slides. The cells are fixed by choosing a  
fixative which provides the best spatial resolution of the  
cells and the optimal hybridization efficiency. After  
20 fixation, the support bound cells may be dehydrated and  
stored at room temperature or the hybridization procedure  
may be carried out immediately.

The hybridization step is then carried out in a  
solution containing a chaotropic agent such as 50%  
25 formamide, a hybrid stabilizing agent such as five times  
concentrated SSC solution (1x = 0.15M sodium chloride and  
0.015M sodium citrate), a buffer such as 0.1M sodium  
phosphate (pH 7.4), about 100 micrograms (ug)/milliliter  
(ml) low molecular weight DNA to diminish non-specific  
30 binding, 0.1% Triton X-100 to facilitate probe entry into  
the cells and about 10-20 mM vanadyl ribonucleoside  
complexes.

To the hybridization solution is added a probe,  
to hybridize with a target polynucleotide. The most  
35 preferable probe is a single-stranded RNA probe,

1 approximately 75 to 150 bases in length. An antibody  
probe may be utilized to bind to a target protein or  
antigen. The hybridization solution containing the probe  
is added in an amount sufficient to cover the cells. The  
5 cells are then incubated at 55°C for at least 30 minutes.  
The probe is added at a high concentration of at least  
about 1 ug/ml of hybrid mix in order to give optimal  
results in this time frame.

The probes may be detectably labeled prior to  
10 addition to the hybridization solution. Alternatively, a  
detectable label may be selected which binds to the  
hybridization product. Probes may be labeled with any  
detectable group for use in practicing the invention.  
Such detectable group can be any material having a  
15 detectable physical or chemical property. Such detectable  
labels have been well-developed in the field of  
immunoassays and in general most any label useful in such  
methods can be applied to the present invention.  
Particularly useful are enzymatically active groups, such  
20 as enzymes (see Clin. Chem., 22:1243 (1976)), enzyme  
substrates (see British Pat. Spec. 1,548,741), coenzymes  
(see U.S. Patents Nos. 4,230,797 and 4,238,565) and enzyme  
inhibitors (see U.S. Patent No. 4,134,792); fluorescers  
(see Clin. Chem., 25:353 (1979); chromophores; luminescers  
25 such as chemiluminescers and bioluminescers (see Clin.  
Chem., 25:512 (1979)); specifically bindable ligands;  
proximal interacting pairs; and radioisotopes such as  
 $^3\text{H}$ ,  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^{125}\text{I}$  and  $^{14}\text{C}$ .

The invention of the present application which  
30 provides optimal fixatives allowing probe entry and  
blocking of non-specific probe binding and formamide  
hybridization at high temperatures (55°C) provides a  
hybridization assay with rapid kinetics of hybrid  
formation and sensitivity of as few as 1-5 molecules per  
35 cell.

1           The superior results of the invention of the  
present application is postulated to occur by preventing  
precipitation of cellular constituents onto mRNA or the  
covalent modification of mRNA, the destabilization of  
5       ribosomal RNA subunit binding, and promotion of  
accessibility of full length mRNA for hybrid formation by  
inducing single-strandedness in cellular RNA and/or DNA.  
The present invention arose out of the applicant's  
discovery of the strong correlation between cellular RNA  
10       single-strandedness and the rapid kinetics of  
hybridization which yielded a highly sensitive assay  
procedure.

In one aspect, the present invention provides a  
simple method to determine the optimal fixation/  
15       prehybridization/hybridization/detection conditions for  
any tissue type so that: (1) single molecules may be  
detected, (2) cellular morphology will be preserved and  
(3) the total reaction time will be reduced to 2-4 hours.

Briefly, in order to predict the optimal  
20       conditions to achieve this rapid and sensitive  
hybridization, a cellular specimens in multiple samples,  
either in suspension or deposited on glass slides, are  
exposed first to a fixative and subsequently to a  
hybridization solution.

25       The fixative is selected from the group  
consisting of 95% ethanol/5% acetic acid, 75% ethanol/20%  
acetic acid, 50% methanol/50% acetone and 10%  
formaldehyde/90% methanol (all v/v). Other useful  
fixatives will be obvious to one skilled in the art as  
30       long as the fixative selected allows at least a 70% shift  
of double stranded to single stranded cellular  
polynucleotides while maintaining cellular spatial  
relationships. The duration of exposure to the fixative  
is from 1 to 180 min. Preferably, 1 to 30 min., and most  
35       preferably 20 min. The temperature of the fixation



1 procedure is preferably -20 to 50°C. and most preferably  
20°C. A subsequent exposure to 70% ethanol/30% water for  
0.5 min. to 20 min. at -20 to 30°C. may be utilized if  
samples are to be stored prior to hybridization.

5 The hybridization solution consists of a  
chaotropic denaturing agent, a buffer, a pore forming  
agent, a hybrid stabilizing agent, non-specific  
nucleotides, and a target specific probe.

10 The chaotropic denaturing agent (Robinson, D. W.  
and Grant, M. E. (1966) J. Biol. Chem. 241: 4030;  
Hamaguchi, K. and Geiduscheck, E. P. (1962) J. Am. Chem.  
Soc. 84: 1329) is selected from the group consisting of  
formamide, urea, thiocyanate, guanidine, trichloroacetate,  
tetramethylamine, perchlorate, and sodium iodide. Any  
15 buffer which maintains pH at least between 7.0 and 8.0 may  
be utilized.

20 The pore forming agent is for instance, a  
detergent such as Brij 35, Brij 58, sodium dodecyl  
sulfate, CHAPS<sup>TM</sup> Triton X-100. Depending on the  
location of the target biopolymer, the pore-forming agent  
is chosen to facilitate probe entry through plasma, or  
nuclear membranes or cellular compartmental structures.  
For instance, 0.05% Brij 35 or 0.1% Triton X-100 will  
permit probe entry through the plasma membrane but not the  
25 nuclear membrane. Alternatively, sodium desoxycholate  
will allow probes to traverse the nuclear membrane. Thus,  
in order to restrict hybridization to the cytoplasmic  
biopolymer targets, nuclear membrane pore-forming agents  
are avoided. Such selective subcellular localization  
30 contributes to the specificity and sensitivity of the  
assay by eliminating probe hybridization to complimentary  
nuclear sequences when the target biopolymer is located in  
the cytoplasm. Agents other than detergents such as  
fixatives may serve this function. Furthermore, a  
35 biopolymer probe may also be selected such that its size

1 is sufficiently small to traverse the plasma membrane of a cell but is too large to pass through the nuclear membrane.

Hybrid stabilizing agents such as salts of mono- and di-valent cations are included in the hybridization solution to promote formation of hydrogen bonds between  
5 complimentary sequences of the probe and its target biopolymer. Preferably sodium chloride at a concentration from .15M to 1M is used; most preferably, the concentration of sodium chloride is 0.6M.

10 In order to prevent non-specific binding of nucleic acid probes, nucleic acids unrelated to the target biopolymers are added to the hybridization solution at a concentration of 100 fold the concentration of the probe.

Specimens are removed after each of the above  
15 steps and analyzed by observation of cellular morphology as compared to fresh, untreated cells using a phase contrast microscope. The condition determined to maintain the cellular morphology and the spatial resolution of the various subcellular structures as close as possible to the  
20 fresh untreated cells is chosen as optimal for each step.

In addition, cellular nucleic acids were stained with about 50 ug/ml propidium iodide dye. This dye has a specific characteristic fluorescent emission (about 480 nm, green) when the nucleic acid is single-stranded and  
25 emits at a different wave length (about 615 nm, red) when the nucleic acid is double-stranded. The dye utilized may be dependent upon whether the target sequence for the particular assay is RNA or DNA. If the assay is to detect low copy numbers of DNA, then a DNA detecting dye such as  
30 acridine orange, tetrahydrofuran, methyl green, pyronin Y and azure B are used, and the nuclear DNA is analyzed for the amount of single or double-strandedness. If instead, the assay is to be used to detect low copy numbers of RNA, then RNA dye such as Acridines, Azines, Xanthenes,  
35 Oxazines, and Thiazines are used and the cytoplasmic RNA

1 is analyzed for the amount of single or  
double-strandedness. Regardless of whether the assay is  
used to analyze RNA or DNA, the optimal conditions are  
reached when the nucleic acid to be detected has undergone  
5 a 70% shift from double-strandedness to  
single-strandedness. When the shift of the secondary  
structure of the nucleic acid from double-strandedness to  
single-strandedness has reached at least 70%, and there is  
no decrease in the total amount of fluorescence, then the  
10 conditions have been adjusted according to the present  
invention and will permit optimal hybridization and  
detection of as few as 1-5 molecules of target nucleic  
acid within a single cell. Furthermore, the time required  
for optimal hybridization can be determined from the  
15 amount of time necessary for at least 70% of the cellular  
nucleic acid to become single-stranded.

In the most preferred embodiment, the  
hybridization assay of the present invention provides a  
method for assaying biopolymers in a cell sample having  
20 substantially intact membranes comprising the steps of  
1) depositing the target cells onto a solid support,  
2) fixing the cells, 3) incubating the cells with a  
hybridization solution containing a single-stranded RNA  
probe, 4) detecting the amount of probe hybridized to the  
25 target nucleic acid. The samples are then washed and the  
amount of target nucleic acids are determined by  
quantitation either photographically through a microscope  
with fluorescent capabilities or by direct reading of the  
fluorescence with a Meridian ACAS 470 work station  
30 (Meridian Instruments, Okemos, Michigan).

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 demonstrates the optimal temperature of  
the In Situ Hybridization.

Figure 2 demonstrates the kinetics of the In Situ

1 Hybridization reaction.

Figure 3 demonstrates the changes in secondary structure of cellular RNA as a function of efficiency of the In Situ Hybridization reaction.

5 Figure 4 demonstrates the sensitivity of the In Situ Hybridization reaction using a control cell line, Balb/c3T3.

Figure 5 demonstrates the detection of oncogenes in normal peripheral blood, normal bone marrow and chronic myelogenous leukemia (CML) by In Situ Hybridization.

10 Figure 6 demonstrates the detection of oncogenes in solid tissue samples by In Situ Hybridization.

Figure 7 demonstrates the specificity of detection of Human Immunodeficiency Virus (HIV) in positive and negative controls by In Situ Hybridization.

15 Figure 8 demonstrates the detection of HIV in patients with Kaposi Sarcoma (KS) or AIDS Related Complex (ARC) by In Situ Hybridization.

Figure 9 demonstrates the detection of HIV in patients with Acquired Immune Deficiency Syndrome (AIDS) or Lymphoma by In Situ Hybridization.

20 Figure 10 demonstrates the detection of HIV in seropositive (Ab+), asymptomatic, high risk individuals by In Situ Hybridization.

25 Figure 11 demonstrates the simultaneous detection by In Situ Hybridization of three oncogenes within the same peripheral blood cells of a patient with chronic myelogenous leukemia (CML). Fluorescent and enzymatic In Situ Hybridization detections are used for the analysis.

30 Figure 12 demonstrates the simultaneous detection of three oncogenes within the same peripheral blood cells of a patient with chronic myelogenous leukemia (CML). Fluorescent and colloidal gold In Situ Hybridization detections are used for the analysis.

35 Figure 13 demonstrates the simultaneous detection

1 of antigens and nucleic acids within the same cells using  
In Situ Hybridization.

Figure 14 demonstrates a quantitative analysis  
of In Situ Hybridization data.

5 Figure 15 demonstrates the detection of  
Cytomegalovirus (CMV) in patients with Kaposi Sarcoma  
(KS), AIDS Related Complex (ARC), Acquired Immune  
Deficiency Syndrome (AIDS), or Lymphoma.

10 Figure 16 demonstrates the detection of  
Cytomegalovirus (CMV) in seropositive (Ab+), asymptomatic,  
high risk individuals by In Situ Hybridization.

15 Figure 17 demonstrates the detection by In Situ  
Hybridization of four different portions of HIV (GAG, ENV,  
TAT, LTR) in a person who is at risk for viral infection  
but tests sero-negative for HIV.

Figure 18 demonstrates the confirmation of the In  
Situ Hybridization results in Figure 17 by a Southern Blot  
assay.

20 Figure 19 demonstrates the ability to monitor the  
results of alpha-interferon therapy in patients by In Situ  
Hybridization.

Figure 20 demonstrates the ability to monitor the  
results of gamma-interferon therapy in patients by In Situ  
Hybridization.

## 25 DETAILED DESCRIPTION OF THE INVENTION

### Mounting Cells/Tissues

30 The first step in the in situ hybridization  
procedure is the deposition of specimens onto a solid  
support. Specimens constitute any material which is  
composed of or contains cells or portions of cells. The  
cells may be living or dead, so long as the target  
biopolymer (DNA, RNA or protein) is unaltered and  
undamaged and capable of detection. The specimen should  
35 contain cells with substantially intact membranes.

1 Although it is not necessary that all membranes of the  
cellular structure be intact, the membranes must be  
sufficiently preserved to allow: retention of the target  
biopolymer, introduction of the detecting probe to the  
5 site of the target biopolymer and preservation of  
antigenicity of any target membrane components.

Techniques for depositing the specimens on the  
solid support will depend upon the cell or tissue type and  
may include, for example, standard sectioning of tissue or  
10 smearing or cytocentrifugation of single cell  
suspensions.

Many types of solid supports may be utilized to  
practice the invention. Supports which may be utilized  
include, but are not limited to, glass, Scotch tape (3M),  
15 nylon, Gene Screen Plus (New England Nuclear) and  
nitrocellulose. Most preferably glass microscope slides  
are used. The use of these supports and the procedures  
for depositing specimens thereon will be obvious to those  
of skill in the art. The choice of support material will  
depend upon the procedure for visualization of cells and  
20 the quantitation procedure used. Some filter materials  
are not uniformly thick and, thus, shrinking and swelling  
during in situ hybridization procedures is not uniform.  
In addition, some supports which autofluoresce will  
25 interfere with the determination of low level  
fluorescence. Glass microscope slides are most  
preferable as a solid support since they have high  
signal-to-noise ratios and can be treated to better retain  
tissue.

### 30 Fixation of Cells/Tissues

After depositing cells or sections on solid  
supports, the samples are fixed. A fixative may be  
selected from the group consisting of any precipitating  
agent or cross-linking agent used alone or in combination,  
35 and may be aqueous or non-aqueous. The fixative may be

1 selected from the group consisting of formaldehyde  
solutions, alcohols, salt solutions, mercuric chloride  
sodium chloride, sodium sulfate, potassium dichromate,  
potassium phosphate, ammonium bromide, calcium chloride,  
5 sodium acetate, lithium chloride, cesium acetate, calcium  
or magnesium acetate, potassium nitrate, potassium  
dichromate, sodium chromate, potassium iodide, sodium  
iodate, sodium thiosulfate, picric acid, acetic acid,  
paraformaldehyde, sodium hydroxide, acetones, chloroform,  
10 glycerin, thymol, etc. Preferably, the fixative will  
comprise an agent which fixes the cellular constituents  
through a precipitating action and has the following  
characteristics: the effect is reversible, the cellular  
morphology is maintained, the antigenicity of desired  
15 cellular constituents is maintained, the nucleic acids are  
retained in the appropriate location in the cell, the  
nucleic acids are not modified in such a way that they  
become unable to form double or triple stranded hybrids,  
and cellular constituents are not affected in such a way  
20 so as to inhibit the process of nucleic acid hybridization  
to all resident target sequences. Choice of fixatives and  
fixation procedures can affect cellular constituents and  
cellular morphology; such effects can be tissue specific.  
Preferably, fixatives for use in the invention are  
25 selected from the group consisting of ethanol,  
ethanol-acetic acid, methanol, and methanol-acetone which  
fixatives afford the highest hybridization efficiency with  
good preservation of cellular morphology.

Fixatives most preferable for practicing the  
30 present invention include 95% ethanol/5% acetic acid for  
HL-60 and normal bone marrow cells, 75% ethanol/20% acetic  
acid for K562 and normal peripheral blood cells, 50%  
methanol/50% acetone for fibroblast cells and normal bone  
marrow cells, and 10% formaldehyde/90% methanol for  
35 cardiac muscle tissue. These fixatives provide good

1 preservation of cellular morphology and preservation and  
accessibility of antigens, and high hybridization  
efficiency. According to the present invention, one or  
two fixatives for each tissue type are provided which  
5 ensure both the best spatial resolution of cells and the  
optimal hybridization efficiency.

Simultaneously, the fixative may contain a  
compound which fixes the cellular components by  
cross-linking these materials together, for example,  
10 glutaraldehyde or formaldehyde. While this cross-linking  
agent must meet all of the requirements above for the  
precipitating agent, it is generally more "sticky" and  
causes the cells and membrane components to be secured or  
sealed, thus, maintaining the characteristics described  
15 above. The cross linking agents when used are preferably  
less than 10% (v/v).

Cross-linking agents, while preserving  
ultrastructure, often reduce hybridization efficiency;  
they form networks trapping nucleic acids and antigens and  
20 rendering them inaccessible to probes and antibodies.  
Some also covalently modify nucleic acids preventing later  
hybrid formation.

#### Storage of Cells/Tissues

After fixation, microscope slides containing  
25 cells may be stored air dried at room temperature for up  
to three weeks, in cold (4°C) 70% ethanol in water for  
6-12 months, or in paraplast for up to two years. If  
specimens are handled under RNase free conditions, they  
can be dehydrated in graded alcohols and stored for at  
30 least 5 months at room temperature.

#### Prehybridization Treatments

According to the present invention no formal  
prehybridization step is necessary. Blocking nonspecific  
binding of probe and facilitating probe entry can be  
35 accomplished in the hybridization solution. If short



1        hybridizations are to be done (> 30 min.), slides may be  
preheated to hybridization temperature before addition of  
the hybridization solution.

#### Hybridizations

5                Nucleic acid hybridization is a process where two  
or more mirror images or opposite strands of DNA, RNA,  
oligonucleotides, polynucleotides, or any combination  
thereof recognize one another and bind together through  
the formation of some form of either spontaneous or  
10        induced chemical bond, usually a hydrogen bond. The  
degree of binding can be controlled based on the types of  
nucleic acids coming together, and the extent of "correct"  
binding as defined by normal nucleic acids coming  
together, and the extent of "correct" binding as defined  
15        by normal chemical rules of bonding and pairing. For  
example, if the binding of two strands forms 9 out of 10  
correct matches along a chain of length 10, the binding is  
said to be 90% homologous.

20                Cellular nucleic acid sequences are detected by  
the process of molecular hybridization. The probe must be  
"labeled" in some way so to allow "detection" of any  
complementary cellular nucleic acid sequences present  
within the individual cells.

25                In the present invention, the term  
"hybridization" also means the binding of an antibody to a  
target antigen.

#### Types of Probes

30                A probe is defined as genetic material DNA, RNA,  
or oligonucleotides or polynucleotides comprised of DNA or  
RNA and antibodies. The DNA or RNA may be composed of the  
bases adenosine, uridine, thymidine, guanine, cytosine, or  
any natural or artificial chemical derivatives thereof.  
The probe is capable of binding to a complementary or  
mirror image target cellular genetic sequence through one  
35        or more types of chemical bonds, usually through hydrogen

1 bond formation. The extent of binding is referred to as  
the amount of mismatch allowed in the binding or  
hybridization process; the extent of binding of the probe  
to the target cellular sequences also relates to the  
5 degree of complementarity to the target sequences. The  
size of the probe is adjusted to be of such size that it  
forms stable hybrids at the desired level of mismatch;  
typically, to detect a single base mismatch requires a  
probe of approximately 12-50 bases. Larger probes (from  
10 50 bases up to tens of thousands of bases) are more often  
used when the level of mismatch is measured in terms of  
overall percentage of similarity of the probe to the  
target cellular genetic sequence. The size of the probe  
may also be varied to allow or prevent the probe from  
15 entering or binding to various regions of the genetic  
material or of the cell. Similarly, the type of probe  
(for example, using RNA versus DNA) may accomplish these  
objectives. The size of the probe also affects the rate  
of probe diffusion, probability of finding a cellular  
20 target match, etc. Typically, double-stranded DNA  
(dsDNA), single-stranded DNA (ssDNA) or RNA probes are  
used in a hybridization reaction when nucleotide sequences  
are the target.

Nucleic acid probes can be prepared by a variety  
25 of methods known to those of skill in the art. Purified  
double-stranded sequences of DNA (dsDNA) can be labeled  
intact by the process of nick translation or random primer  
extension. The ability of double-stranded probes to  
hybridize to nucleic acids immobilized within cells is  
30 compromised by the ability of the complementary strands to  
hybridize to each other in solution prior to hybridization  
with the cellular nucleic acids. Single-stranded DNA  
(ssDNA) probes do not suffer this limitation and may be  
produced by the synthesis of oligonucleotides, by the use  
35 of the single-stranded phage M13 or plasmid derivatives of

1 this phage, or by reverse transcription of a purified RNA  
template. The use of single-stranded RNA (ssRNA) probes  
in hybridization reactions potentially provides greater  
signal-to-noise ratios than the use of either double or  
5 single-stranded DNA probes. Regardless of whether a  
dsDNA, a ssDNA, or a ssRNA probe is used in the  
hybridization reaction, there must be some means of  
detecting hybrid formation. The means of detecting hybrid  
formation utilizes a probe "labeled" with some type of  
10 detectable label.

Antibody probes are known to those skilled in the  
art. The term "antibody probe" means an antibody that is  
specific for and binds to any target antigen. Such a  
target antigen may be a peptide, protein, carbohydrate or  
15 any other biopolymer to which an antibody will bind with  
specificity.

#### Detection Systems

Detectable labels may be any molecule which may  
be detected. Commonly used detectable labels are  
20 radioactive labels including, but not limited to,  $^{32}\text{P}$ ,  
 $^{14}\text{C}$ ,  $^{125}\text{I}$ ,  $^3\text{H}$  and  $^{35}\text{S}$ . Biotin labeled nucleotides  
can be incorporated into DNA or RNA by nick translation,  
enzymatic, or chemical means. The biotinylated probes are  
detected after hybridization using avidin/streptavidin,  
25 fluorescent, enzymatic or colloidal gold conjugates.  
Nucleic acids may also be labeled with other fluorescent  
compounds, with immunodetectable fluorescent derivatives  
or with biotin analogues. Nucleic acids may also be  
labeled by means of attaching a protein. Nucleic acids  
30 cross-linked to radioactive or fluorescent histone H1,  
enzymes (alkaline phosphatase and peroxidases), or  
single-stranded binding (ssB) protein may also be used.  
To increase the sensitivity of detecting the colloidal  
gold or peroxidase products, a number of enhancement or

1        amplification procedures using silver solutions may be  
      used.

      An indirect fluorescent immunocytochemical  
      procedure may also be utilized (Rudkin and Stollar (1977)  
5        Nature 265: 472; Van Prooijen, et al (1982) Exp.Cell.Res.  
      141: 397). Polyclonal antibodies are raised against  
      RNA-DNA hybrids by injecting animals with  
      poly(rA)-poly(dT). DNA probes were hybridized to cells in  
      situ and hybrids were detected by incubation with the  
10        antibody to RNA-DNA hybrids.

      According to the present invention RNA probes are  
      preferable to DNA probes (5-8 fold more efficient).  
      Labeling probes with Photobiotin<sup>TM</sup> instead of biotin  
      increased the sensitivity of the assay another 2-3 fold.  
15        Probe Size and Concentration

      The length of a probe affects its diffusion rate,  
      the rate of hybrid formation, and the stability of  
      hybrids. According to the present invention, small probes  
      (50-150 bases) yield the most sensitive, rapid and stable  
20        system. A mixture of short probes (50-150 bases) are  
      prepared which span the entire length of the target  
      biopolymer to be detected. For example, if the target  
      biopolymer were 1000 bases long, about 10-20 "different"  
      probes of 50-100 bases would be used in the hybrid  
25        solution to completely cover all regions of the target  
      biopolymer.

      The concentration of the probe affects several  
      parameters of the in situ hybridization reaction. High  
      concentrations are used to increase diffusion, to reduce  
30        the time of the hybridization reaction, and to saturate  
      the available cellular sequences. According to the  
      present invention, the reaction is complete after 30  
      minutes (see Figure 2). To achieve rapid reaction rates  
      while maintaining high signal-to-noise ratios, probe  
35        concentrations of 2.5-5.0 ug/ml are preferable. Most

1 preferable is use of probes at a concentration of 2.5  
ug/ml.

#### Hybridization Solution and Temperature

5 In a preferred embodiment, the hybridization  
solution of the present invention consists of 50%  
formamide, 4X SSC (1X SSC = 0.15M sodium chloride and  
0.015M sodium citrate), about 0.1M sodium phosphate (pH  
7.4), about 100 ug/ml low molecular weight DNA, 0.1%  
10 Triton X-100 and about 10-20mM vanadyl ribonucleoside  
complexes. Single-stranded RNA probe is added to this  
solution. The probe may be at least 15-20 bases,  
preferably, 75-150 bases, and labeled with  
Photobiotin<sup>TM</sup>. As shown in Figure 1 the most preferable  
optimal temperature of hybridization is 50°-55°C.  
15 However, temperatures ranging from 15°C. to 80°C. may be  
used.

#### Post-Hybridization Treatments and Detections

20 The present invention does not require wash steps  
prior to hybrid detections. Instead, avidin or  
streptavidin fluorescent, enzymatic or colloidal gold  
complexes may be added directly to the slides containing  
hybridization cocktail and incubated for 20 minutes at  
room temperature, or 10 minutes at 37°C. This step  
constitutes a significant advantage over prior  
25 hybridization techniques due to the time saved by  
eliminating several post-hybridization washing steps and  
the necessary re-blocking of non-specific  
avidin/streptavidin binding sites; it results in no  
decrease in signal or increase in noise.

30 The streptavidin/avidin detection step is  
followed by washes in large volumes of 2x SSC/0.1% Triton  
X-100. The solution may contain RNase A and T1 at room  
temperature. This wash can be very short (less than 5  
minutes) as long as a continuous gentle circulation or  
35 stream of sufficient volume (about 1-200 ml per cm<sup>2</sup> area

1 of cells) of solution passes over the cells. This may be  
followed by washes at higher stringency (lower salt  
concentrations such as at least 0.1x SSC and/or higher  
temperatures up to 65° C.). Leaving the cell area moist,  
5 supports are then dried and coverslipped by any  
conventional method.

Analysis of the Results of In Situ Hybridizations  
Speed, Sensitivity and Quantitation of In Situ  
10 Hybridizations

The method of the present invention requires 2-4  
hours to complete with a sensitivity of as few as 1-5  
molecules of target biopolymers per cell. This results  
from the combination of at least three factors: 1)  
15 cellular constituents are not irreversibly precipitated  
onto the nucleic acids, 2) the fixation was optimized for  
the particular tissue used, and 3) the kinetics of the  
reaction proceed more rapidly at high probe concentrations  
and at elevated temperatures.

20 The number of copies of mRNA per cell can be  
estimated from the number of grains over cells when  
radioactive probes are used. With fluorescent or  
enzymatic detections a relative estimate of fluorescence  
or precipitated colored products allows estimation of mRNA  
25 copy number. Usually, the approximation of copy number is  
easier after manual photography, film processing and  
comparisons of photographic prints.

The quantitation of radioactive or fluorescent  
signals obtained after in situ hybridizations may be  
30 automated by use of an image analysis system, such as the  
Meridian ACAS 470 workstation as is demonstrated in  
Example 11.

Simultaneous Detection of Three mRNAs

35 The present invention allows simultaneous  
detection of different substances (mRNAs and proteins)

1 within the same cells. This may be accomplished in one of  
two ways. First, multiple probes each containing a unique  
label (for example, fluorescent tags "A", "B" and "C"  
which each emit light at a different detectable wave  
5 length) are all added together in the hybridization  
solutions. Alternatively, a hybridization and detection  
reaction may be carried out with one probe and label,  
residual unreacted probe and label washed away under  
nuclease-free conditions, and another hybridization  
10 reaction is carried out. This process is repeated as many  
times as desired. Example 9 demonstrates one embodiment  
of the detection of multiple target biopolymers in the  
same cell.

The following examples are offered by way of  
15 illustration and are not intended to limit the invention  
in any manner. In all examples, all percentages are by  
weight if for solids and by volume if for liquids, and all  
temperatures are in degrees Celcius unless otherwise  
noted.

20

### EXAMPLE 1

#### Preparation of Probes.

##### 25 A. General.

RNA or DNA probes useful in the present  
invention may be prepared according to methods known to  
those of skill in the art or may be obtained from any  
commercial source. RNA probes may be prepared by the  
methods described by Green et al. (1981) Cell 32:681. DNA  
30 probes may be prepared by methods known to those of skill  
in the art such as described by Rigby et al. (1977) J.  
Mol. Biol. 113:237. Synthetic oligonucleotide probes may  
be prepared as described by Wallace, et al (1979) Nucleic  
Acids Research 6:3543. The probes useful in the present  
35

invention must have the following characteristics:

1. Specificity for the target molecule, and
2. At least 15 base pairs in length and preferably 75-150 base pairs.

B. Preparation of RNA probes.

Sub genomic fragments of the c-myc, c-sis, or c-abl genes were obtained from Amersham Inc. (Catalogue nos. RPN.1315X, RPN.1324X, and RPN.1325X, respectively). In one embodiment of the present invention, sense strand probe of the c-myc, c-abl and c-sis genes were utilized. The c-myc probe used was a 1.3 kb ClaI/EcoRI genomic clone from the 3' end of the c-myc gene (Dalla-Favera, et al. (1983) Science 219:963). The c-abl probe was derived from a subclone of the human c-abl gene, an EcoRI/Bam HI fragment corresponding to the 5' c-abl hybridizing region (de Klein et al. (1982) Nature 300:765). The c-sis probe was a Bam HI fragment of clone L33 corresponding to the 3' end of c-sis (Josephs et al. (1983) Science 219:503). The HIV and EBV probes were obtained from and prepared as described in Dewhurst, et al. (1987) FEBS Lett. 213:133. The CMV probe was described in Gronczol, et al. (1984) Science 224:159. These template plasmid DNAs were transcribed as described by Green et al. (1981) Cell 32:681. The size and quantity of the RNA was confirmed by electrophoresis through a denaturing agarose gel as described by Thomas (1980) Proc. Nat. Acad. Sci. USA 77:5201 and spectrophotometric measurement performed at A260 and A280. A DNA beta-actin probe prepared as described in Cleveland, et. al. (1980) Cell 20:95 and the RNA probes were labeled with Photobiotin™ as described by Bresser and Evinger-Hodges (1987) Gene Anal. Tech. 4: 89, incorporated herein by reference.

Low-molecular weight DNA was added at a concentration of 100 times that of the probe, and all



1 polynucleotides were precipitated by the addition of 1/3  
vol. 10M ammonium acetate and 2-1/2 vol. of 95% ethanol.  
The nucleic acids were recovered by centrifugation and  
resuspended in water at a concentration of 1 microgram  
5 (ug)/microliter (ml) of probe and stored at -70°C until  
used.

### C. Preparation of Antibody Probes

Antibody probes specific for antigens such as  
viruses or specific determinants thereof, peptides and  
10 proteins derived from a variety of sources, carbohydrate  
moieties and a wide variety of biopolymers are known to  
those of skill in the art. The methods for preparation of  
such antibodies are also known to those of skill in the  
art.

15 Briefly, polyclonal antibodies may be prepared by  
immunization of an animal host with an antigen.  
Preferably, the antigen is administered to the host  
subcutaneously at weekly intervals followed by a booster  
dose one month after the final weekly dose. Subsequently,  
20 the serum is harvested, antibodies precipitated from the  
serum and detectably labeled by techniques known to those  
of skill in the art.

Monoclonal antibodies may be prepared according  
to any of the methods known to those in the art. Fusion  
25 between myeloma cells and spleen cells from immunized  
donors has been shown to be a successful method of  
producing continuous cell lines of genetically stable  
hybridoma cells capable of producing large amounts of  
monoclonal antibodies against target antigens such as, for  
instance, tumors and viruses. Monoclonal antibodies may  
30 be prepared, for instance, by the method described in U.S.  
Patent No. 4,172,124 to Koprowski, et al. or according to  
U.S. Patent No. 4,196,265 to Koprowski, et al.

Procedures for labeling antibodies are known to  
35 those of skill in the art.

1

## EXAMPLE 2.

Temperature effect on Hybridization.

5 K562 cells (ATCC # CCL 243) were grown in Hank's Balanced Salt Solution supplemented with 10% fetal calf serum. Dividing cells were deposited onto glass slides by cytocentrifugation. Cells were fixed with 75% ethanol, 20% glacial acetic acid, 5% water for 20 minutes at room  
10 temperature. No prehybridization step was performed. Twenty microliters of hybridization solution consisting of 50% formamide, 4x SSC, 0.1M sodium phosphate (pH 7.4), 0.1% Triton X-100, 100 ug/ml low molecular weight DNA (sheared herring sperm DNA obtained from Sigma Chemical  
15 Company) and 2.5 ug/ml of either c-myc, c-abl or c-sis anti-sense RNA probe labeled with Photobiotin" was added to each specimen. The anti-sense RNA probes were prepared as described in Example 1. The hybridization reactions were carried out at various temperatures ranging from 4° to 80° C. After incubation at the desired temperatures  
20 for two hours, hybrid formation was detected. To detect hybridization, streptavidin fluorescein or rhodamine complexes at 2x the manufacturer's recommended concentration was added to this specimen. After incubation at room  
25 temperature for 30 min the specimens were then gently washed with 1 to 200 ml per centimeter square of cell area with each of the following solutions containing 0.1% Triton X-100, in order: 2xSSC, 1xSSC, 0.5xSSC and 0.1xSSC. One drop of a 50/50 (v/v) 100% glycerol/2x PBS  
30 solution was added to each specimen. Using a Nikon fluorescent microscope with photomultiplier tube attachments the fluorescence emitted per cell was recorded on each slide hybridized at a different temperature. Approximatley 300 to 800 cells were analyzed per slide.  
35 Numerical results obtained indicating the amount of

1 fluorescence from each cell were graphically represented  
as relative fluorescence verses the temperature of  
hybridization.

5 The results shown of Figure 1 demonstrate that  
hybridization temperatures of 50°C to 55°C yields the most  
relative fluorescence corresponding to the most hybrid  
formation in the present in situ hybridization invention.

10 EXAMPLE 3.  
Kinetics of In Situ Hybridization.

Figure 2 shows the relationship between the time  
of hybridization and the amount of fluorescent signal seen  
over cells. K562 cells (ATCC # CCL 243) were grown in  
15 Hank's Balanced Salt Solution supplemented with 10% fetal  
calf serum. Dividing cells were deposited onto glass  
slides by cytocentrifugation. Cells were fixed with 75%  
ethanol, 20% glacial acetic acid, 5% water for 20 minutes  
at room temperature. No prehybridization step was  
20 performed. Twenty microliters of hybridization solution  
consisting of 50% formamide, 4x SSC, 0.1M sodium phosphate  
(pH 7.4), 0.1% Triton X-100, 100 ug/ml low molecular  
weight DNA (sheared herring sperm DNA obtained from Sigma  
Chemical Company) and 2.5 ug/ml of either c-myc, c-abl or  
25 c-sis anti-sense RNA probe labeled with Photobiotin<sup>TM</sup>  
was added to each specimen. The anti-sense RNA probes  
were prepared as described in Example 1. The  
hybridization reactions were carried out at various times  
ranging from 5 minutes to 96 hours. After incubation at  
30 55°C. for the desired time, hybrid formation was  
detected. To detect hybridization, streptavidin  
fluorescein or rhodamine complexes at 2x the manufacturers  
concentration were added to the specimen. After  
incubation at room temperature for 30 minutes the  
35 specimens were then gently washed with 0.1x SSC/0.1%

1 Triton X-100 at 1-200 ml per cm<sup>2</sup> of cell area. One drop  
of a 50/50 (v/v) 100% glycerol/2x PBS solution was added  
to each specimen. Using a Nikon fluorescent microscope  
with photomultiplier tube attachments, the fluorescence  
5 emitted per cell was recorded on each slide hybridized at  
each different time point. Approximately 300 to 800 cells  
were analyzed per slide. Numerical results obtained  
indicating the amount of fluorescence from each cell were  
graphically represented as relative fluorescence versus  
10 the time of hybridization. Figure 2 demonstrates that the  
hybridization reaction is essentially complete after 30  
minutes under the conditions of the present invention.

#### EXAMPLE 4.

##### Changes In Secondary Structure Of Cellular RNA.

15 HL60 cells (ATCC # CCL 240) were grown in Hank's  
Balanced Salt Solution (BSS) supplemented with 10% fetal  
calf serum. Cells were harvested and deposited onto glass  
microscope slides by cytocentrifugation. Cells were air  
20 dried on glass slides and stored at room temperature until  
used. Cells are fixed in one of any number of fixatives  
for this type of experiment. Typical fixatives would  
include 70% ethanol, 95% ethanol/5% glacial acetic acid,  
75% ethanol, 20% glacial acetic acid, 100% methanol, 100%  
25 acetone, 50% acetone, 50% methanol, 4% paraformaldehyde,  
2% paraformaldehyde, 10% formaldehyde/90% methanol. After  
cells were fixed in these fixatives at the appropriate  
time and temperature, slides were removed from the  
fixative and stained with Wright Giemsa or hematoxylin and  
30 eosin by standard laboratory methods. Cell morphology was  
assessed by comparing the degree of preservation of  
morphology after fixation to the morphology prior to  
fixation. Fixatives which did not effectively retain  
visual morphology were arbitrarily as rated as +1.  
35 Fixatives which effectively retained cellular morphology

1        were arbitrarily rated as between +1 and +4 with the most  
effective morphologic preservation of cellular morphology  
rated at +4. A second evaluation as to the effective  
preservation of cells by these fixatives was carried out  
5        when it was desirable to detect cellular antigens. In  
this case, cells were removed from the fixatives and  
incubated with an antibody specific for a particular  
target cellular antigen. Again fixatives which  
effectively maintain antigenicity of cellular components  
10        were arbitrarily rated as +4, while fixatives which did  
not effectively maintain perservation of cellular antigens  
were rated lower, the worst as +1. Fixatives which scored  
as +3 or +4 in terms of preservation of cellular  
morphology and/or preservation of cellular antigenicity  
15        were then used in the following steps. Fresh slides  
containing untreated cells were fixed in these fixatives  
and were incubated in hybridization solution containing  
50% formamide, 4x SSC, 0.1 M sodium phosphate, (pH 7.4),  
0.1% Triton X-100, 100 ug/ml low molecular DNA (sheared  
20        herring sperm DNA obtained from Sigma Chemcial Company).  
No biopolymer probe was included in this solution. The  
cells were incubated in hybridization solution at 50°-55°C  
for 5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes. After  
the completion of this hybridization step, cell samples  
25        were washed gently with 1-200 ml per square centimeter of  
cell area with each of the following solutions containing  
0.1% Triton X-100: 2x SSC, 1x SSC, 0.5x SSC, 0.1x SSC.  
The cellular sample was then evaluated as above for  
preservation of cellular morphology and/or preservation of  
cellular antigenicity. The cell sample was then further  
30        evaluated by staining the cells with 50 ug/ml of propidium  
iodide. The propidium iodide will stain double stranded  
and single stranded nucleic acids within the cell. When  
this dye stains double stranded or single stranded nucleic  
acids it has a different characteristic emission spectra  
35

1 upon ultraviolet excitation. An untreated cell sample on  
a slide is also stained. The total amount of emitted  
fluorescence is determined on the untreated cell sample  
using a Nikon fluorescence microscope with a  
5 photomultiplier tube attachment. 300-1000 cells are  
recorded as to the total amount of fluorescence generated  
from cytoplasmic double stranded RNA content. This  
measurement then represents a base line level for the  
total fluorescence in the cytoplasm; that is, the total  
10 RNA in the cytoplasm and that RNA being present in a 100%  
state of double strandedness. The slides which have been  
taken through the various fixation and hybridization  
procedures and times are similarly analyzed. In all cases  
it is important to chose a fixation and hybridization  
15 condition and time which will maintain the same quantity  
of fluorescence in the cytoplasm of the cell. During  
hybridization, the fluorescence emitted from the RNA of  
the cytoplasm of the cell due to the staining of the  
propidium iodide will change. The emission pattern  
20 decreases relative to the double strandedness of the RNA.  
Simultaneously, the wave length emisson which is  
reflective of the amount of single stranded RNA in the  
cytoplasm will begin to increase. When the total  
fluorescence in the cytoplasm due to RNA has remained the  
25 same and the amount of fluorescence due to the amount of  
double stranded RNA in the cytoplasm has decreased  
approximately 70% while the amount of fluorescence  
corresponding to the single stranded RNA within the  
cytoplasm has increased an equal value, then conditions  
30 have been obtained which will allow the detection of 1-5  
molecules of RNA within the cytoplasm. The time of the  
hybridization reaction which was required to obtained this  
shift from double stranded to single strandedness of the  
RNA in the cytoplasm is reflective of the time necessary  
35 for an actual hybridization reaction to detect 1-5

1 molecules per cell of RNA.

Specifically, in Figure 3 the relative amount of double stranded RNA content is graphically represented on the bottom scale. As the RNA in the cytoplasm becomes more double stranded, the more to the right the curves will fall. The greater the shift in the amount of double strandedness to single strandedness of RNA in the cytoplasm, the greater the shift will be of the curves from the right to the left. The vertical axis represents the cell numbers that were counted. In other words if 300-1000 cells were counted, the vast majority of them fell within a particular area of double strandedness. While some cells had more double strandedness and some had less double strandedness, the analysis can be represented as a bell shape curve. On the right hand side of the figure is shown the various treatments carried out. The result of staining untreated cells with propidium iodide is not shown. However, after treating HL60 cells with various fixatives the amount of double strandedness of cellular RNA remained essentially the same. Even if a prehybridization treatment is carried out which includes a protease treatment there is essentially no change in the amount of RNA double strandedness. The curve in Figure 3 corresponding to the protease treatment is in the same location as the curve for the fixation treatment. It has shifted neither left nor right. However, after fifteen minutes in a hybridization solution, the curve representing the amount of RNA double strandedness has shifted at least 70% to the left. This corresponds to a change in at least 70% of the amount of material in the cytoplasm of the cell becoming single stranded. Comparing this graph to Figure 2 indicates that after 15 min. in the hybridization cocktail, not only is 70% of the RNA in the cytoplasm of the cell single stranded, but as seen in Figure 2, 70% of the hybridization reaction is complete.

EXAMPLE 5.Detection of c-myc oncogene.

Balb/c3T3 cells (ATCC #CCL 163) were grown to density arrest in medium on 8-chamber slides (Tissue-Tek, Miles Laboratories). The medium [Hank's Balanced Salt Solution (BSS) supplemented with 10% fetal calf serum (FCS)] was replaced with serum free medium and the cells serum starved overnight. Multiple specimens were then incubated either in the presence or absence of 15% FCS in Hanks BSS for 45 min at 37°C.

Cells were fixed with 50% acetone and 50% methanol for 20 minutes at room temperature.

No prehybridization step was performed. 20 ul of hybridization solution consisting of 50% formamide, 4x SSC, 0.1M sodium phosphate (pH 7.4), 0.1% Triton X-100, 100 ug/ml low molecular weight DNA (sheared herring sperm DNA obtained from Sigma Chemical Co.) and 2.5 ug/ml c-myc anti-sense RNA probe labelled with Photobiotin<sup>TM</sup>, was added to each specimen. The c-myc antisense RNA probe was prepared as described in Example 1. After incubation for 2 hrs. at 55°C, hybrid formation was detected.

To detect hybrids, a streptavidin fluorescein complex (Guesdon, J. L., et al (1979) J. Histochem. Cytochem. 27.1131) at 2x the manufacturer's recommended concentration (Bethesda Research Laboratories; Catalog #9538SA; recommended concentration: 7.5 ug/ml) was added to the specimen. After incubation at room temperature for 30 minutes, the specimens were gently washed sequentially (1-200 ml per cm<sup>2</sup> of cell area) with each of the following solutions containing 0.1% Triton-X 100, in order: 2x SSC, 1x SSC, 0.5x SSC, and 0.1x SSC. One drop of a 50/50 (v/v) 100% glycerol/2x PBS solution was added to each specimen. Specimens were photographed with high speed film (Kodak EES135, PS 800/1600) at 1600 ASA for a 20 sec. exposure on a Nikon Photophot microscope at 400x



1 magnification using a standard filter combination for  
transmission of fluorescent light.

It has been shown by conventional methods (mRNA  
dot blots, Northern blots, and solution hybridizations)  
5 that the c-myc oncogene is not expressed in serum starved  
cells but 1-10 copies per cell are induced in serum  
stimulated cells. (Armelin et al (1984) Nature 310:656).  
Cells probed for expression of c-myc mRNA by the in situ  
hybridization procedure of the present invention are shown  
10 in Figure 4A and Figure 4B. No c-myc mRNA was detected in  
serum starved Balb/c 3T3 cells (Figure 4A) while 1 to 10  
copies of c-myc mRNA was detected in serum stimulated  
cells by the method of the present invention (Figure 4B).

#### 15 EXAMPLE 6.

##### Detection of Oncogenes in Peripheral Blood Cells and Bone Marrow Cells.

Ten ml. of human peripheral blood or 2 ml. of  
human bone marrow cells were incubated at 37° C. in a 1.2%  
20 (215 mOs) ammonium oxalate solution to lyse the red blood  
cells. The white blood cells were centrifuged at 3,000  
rpm for 10 minutes in a clinical centrifuge. The cell  
pellet was subsequently washed with 10 ml. PBS and the  
pellet was resuspended in PBS. Cells were deposited by  
25 cytocentrifugation onto precleaned glass slides and air  
dried for 5 min. The cells were then fixed in 75%  
ethanol/ 20% acetic acid for 20 min. at room temperature.

No prehybridization step was performed. 20 ul of  
hybridization solution consisting of 50% formamide,  
30 4x SSC, 0.1M sodium phosphate (pH 7.4), 0.1% Triton X-100,  
100 ug/ml low molecular weight DNA (sheared herring sperm  
DNA obtained from Sigma Chemical Co.) and 2.5 ug/ml of  
either c-myc, c-sis, or c-abl anti-sense RNA probe  
labelled with Photobiotin <sup>TM</sup>, was added to each  
35 specimen. The anti-sense RNA probes were prepared as

1 described in Example 1. After incubation for 2 hrs. at  
55°C, hybrid formation was detected.

To detect hybrids, streptavidin fluorescein  
complex at 2x the manufacturer's recommended concentration  
was added to the specimen. After incubation at room  
5 temperature for 30 minutes, the specimens were then gently  
washed (1-200 ml per cm<sup>2</sup> of cell area) with each of the  
following solutions containing 0.1% Triton-X 100, in  
order: 2x SSC, 1x SSC, 0.5x SSC, and 0.1x SSC. One drop  
10 of a 50/50 (v/v) 100% glycerol/2x PBS solution was added  
to each specimen. Specimens were photographed with high  
speed film (Kodak EES135, PS 800/1600) at 1600 ASA for  
20 sec. exposure on a Nikon Photophot microscope at 400x  
magnification using a standard filter combination for  
15 transmission of fluorescent light.

Figure 5 depicts the results from in situ  
hybridization studies on the expression of three different  
oncogenes in normal bone marrow (BM), normal peripheral  
blood (PB), or the peripheral blood from patients with  
20 chronic myelogenous leukemia (CML). Blood was obtained  
from these patients when they were either in the chronic  
stage of the disease or in the blast stage of the  
disease. In Figure 5, on the left hand side is shown the  
three different oncogenes which were analyzed, c-sis,  
25 c-myc and c-abl. The numbers below the prints for BM or PB  
indicate the percent of cells in that sample which are  
expressing the oncogene indicated on the left. To the  
bottom of each column of prints (titled BM, PB, chronic or  
blast) is shown a graphic representation of the relative  
30 fluorescent intensity obtained after the hybridizations.  
The relative fluorescent intensity is indicative of the  
amount of RNA present within each cell and is scored on a  
per cell basis. CML in the chronic phase is defined as  
having less than 5% of the cells in the peripheral blood  
35 exist as blasts. In reality, we find that 5-10% of the

1 cells in the peripheral blood are over expressing the  
three genes which were studied. The expression of these  
genes is also considerably elevated as compared to either  
normal BM or PB as seen both in the prints and below the  
5 prints on the graph. In the blast phase of the disease  
which is defined as having greater than 35% of the cells  
in the peripheral blood exist as blasts, we find that  
greater than 70% of the cells are typically expressing the  
three oncogenes c-sis, c-myc, and c-abl. The expression of  
10 these genes is elevated when compared to normal bone  
marrow or normal peripheral blood but is lower than the  
expression of these genes on a per cell basis in the  
chronic phase of the disease as seen both in the prints  
and in the graphic representation.

15

#### EXAMPLE 7.

##### Oncogene detection in solid tissue.

Four micron thick frozen sections of human  
breast tissue obtained from surgically removed biopsy  
20 samples were mounted on precleaned glass slides and fixed  
with 50% methanol/50% acetone for 20 min. at room  
temperature.

Tissue was hybridized for 4 hours by incubation  
at 55°C with a hybridization cocktail containing 50%  
25 formamide, 5x SSC, 0.1 M sodium phosphate (pH 7.4) 20 mM  
vanadyl ribonucleoside complexes (New England Biolabs),  
100 ug/ml of low molecular weight denatured herring sperm  
DNA, and 0.1% Triton X-100. Photobiotinylated RNA probes  
(prepared as described in Example 1) were added to the  
30 hybridization cocktail at a concentration of 2.5 ug/ml. No  
probe was added to the blank panel (Figure 6). Hybrids  
were detected by adding fluorescein labelled  
avidin/streptavidin (A/SA) solutions directly onto the  
slides, and incubated for 30 minutes at room temperature.  
35 Slides were washed, coverslipped and photographed as

described in Example 6.

Figure 6 demonstrates the results of mRNA in situ hybridization and the localization of SIS/PDGF-B expression in the epithelial components of fibrocystic disease (Figure 6, panel "SIS") and lactating adenoma (Figure 6, panel "SIS"). In situ hybridization with a Photobiotinylated DNA probe demonstrating expression of the actin gene in the stroma as well as in the epithelial cells of fibrocystic disease (Figure 6, panel "ACTIN"). Lower panels show comparable phase contrast microscopic features of the tissue.

#### EXAMPLE 8.

##### Detection of HIV in Human Peripheral Blood.

Ten ml. of human peripheral blood or 2 ml. of human bone marrow cells were incubated at 37° C. in a 1.2% ammonium oxalate solution to lyse the red blood cells. The white blood cells were centrifuged at 3,000 rpm for 10 minutes in a clinical centrifuge. The cell pellet was subsequently washed with 10 ml PBS and the pellet was resuspended in PBS. Cells were deposited by cytocentrifugation onto precleaned glass slides and air dried for 5 min. The cells were then fixed in 75% ethanol/ 20% acetic acid for 20 min. at room temperature.

No prehybridization step was performed. 20 ul of hybridization solution consisting of 50% formamide, 4x SSC, 0.1M sodium phosphate (pH 7.4), 0.1% Triton X-100, 100 ug/ml low molecular weight DNA (sheared herring sperm DNA obtained from Sigma Chemical Co.) and 2.5 ug/ml HIV anti-sense RNA probes labeled with Photobiotin<sup>TM</sup>, was added to each specimen. The antisense RNA probes were prepared as described in Example 1. After incubation for 2 hrs. at 55°C, hybrid formation was detected.

To detect hybrids, streptavidin fluorescein complex at 2x the manufacturer's recommended concentration

1 was added to the specimen. After incubation at room  
temperature for 30 minutes, the specimens were then gently  
washed (1-200 ml per cm<sup>2</sup> of cell area) with each of the  
following solutions containing 0.1% Triton-X 100, in  
5 order: 2x SSC, 1x SSC, 0.5x SSC, and 0.1x SSC. One drop  
of a 50/50 (v/v) 100% glycerol/2x PBS solution was added  
to each specimen. Specimens were photographed with high  
speed film (Kodak EES135, PS 800/1600) at 1600 ASA for  
20 sec. exposure on a Nikon Photophot microscope at 400x  
10 magnification using a standard filter combination for  
transmission of fluorescent light.

Figures 7-10 represent the results seen by  
identifying HIV in control cell lines derived from  
patients with AIDS or from fresh patient samples. In  
15 Figure 7 control cell lines either infected with HIV as  
indicated by the plus (+) on the left hand side or a  
control cell line infected by HTLV I as indicated by the  
minus (-) on the left hand side were hybridized to four  
different regions of HIV: ENV, GAG, TAT or LTR genes.  
20 Unless otherwise indicated, anti-sense RNA probes were  
used in the hybridizations. The top four panels indicate  
that these four genes can readily identify HIV in infected  
cells. The control probes, the GAG, ENV, TAT, LTR sense  
probes, do not detect HIV sequences. They are correctly  
25 negative. The anti-sense RNA probes used in the top four  
panels to detect HIV are specific. They do not cross react  
with other viral sequences such as HTLV I, as indicated in  
the bottom two panels. Figure 8 indicates that when the  
GAG and ENV probes are used to detect HIV in patients with  
30 Kaposis Sarcoma (KS), the virus is readily identified in  
fresh peripheral blood. The controls which were performed  
on this blood, sense strand controls for these same genes  
and a Blank (no probe), were, as expected, negative.  
These same probes also identified, as shown in Figure 8, a  
35 virus in a patient with AIDS related complex (ARC). The

1 controls, Sense strand RNA and Blank, are negative. In  
Figure 9, these same anti-sense RNA probes identified HIV  
in a patient with AIDS. The controls, Sense and Blank,  
were negative. In Figure 10 these probes identified the  
5 presence of HIV in an asymptomatic, seropositive (Ab+)  
individual. The controls were negative. These probes did  
not cross react with and did not detect HIV in uninfected  
normal individuals (Figure 10).

#### 10 EXAMPLE 9.

##### Simultaneous Detection of three mRNAs in Human Peripheral Blood.

Fresh peripheral blood from a patient with  
chronic myelogenous leukemia in early accelerated phase  
was obtained by venipuncture. Red blood cells were lysed  
15 with ammonium oxalate. White blood cells were prepared  
and deposited onto slides as described in Example 6.  
After fixation, the specimens were taken through the  
several hybridization steps as described above in  
Example 6 with the following modifications: a probe for  
20 the c-sis gene was incubated with the slides for 1 hour  
using the in situ hybridization solution described in  
Example 5. Streptavidin-rhodamine was used to detect  
hybrid formation. The wash steps followed this detection,  
25 only all solutions were RNase free and contained 0.01M  
D-Biotin. A hybridization was repeated with a second  
probe for the c-myc gene; after 1 hour, the hybrids formed  
were detected using streptavidin-FITC. Washes were  
repeated as above, and a final hybridization was carried  
30 out with a probe for the c-abl gene. Hybrids formed with  
this probe were detected using streptavidin conjugated to  
alkaline phosphatase. The specimens were washed as  
described in Example 5 with the inclusion of 1-10 ug/ml of  
RNase A to each of the wash solutions. The substrates for  
35 the alkaline phosphatase (nitroblue tetrazolium and

1 5-bromo, 4-chloro, 3-indol phosphate) were added, and the  
reduction of nitroblue tetrazolium was carried out for 5  
minutes at room temperature.

Using this in situ hybridization technique which  
5 permitted the simultaneous detection of multiple different  
mRNA species, we have demonstrated that the  
over-expression of c-sis, c-myc and c-abl all occur within  
the same cells in patients' peripheral blood with chronic  
myelogenous leukemia. In Figure 11, the cells containing  
10 the c-myc oncogene mRNA (MYC, the left panel) was detected  
by the presence of a green color emission due to the  
reaction of a streptavidin-fluorescein complex with the  
hybrids formed between the probe and target biopolymer  
sequences. The same cells were also shown to contain the  
15 c-sis oncogene mRNA (SIS, middle panel) by the detection  
of red fluorescence resulting from the presence of reacted  
avidin/streptavidin-rhodamine with the reacted probe. The  
presence of the c-abl oncogene (ABL) within the same cells  
is shown in the right panel by the presence of the dark  
20 blue, reacted and precipitated nitroblue tetrazolium  
product.

In Figure 12, the same result is shown as in  
Figure 11, only a different patient sample was used and a  
different detection method was employed to identify the  
25 presence of the c-abl oncogene mRNA. In this case,  
streptavidin labeled with colloidal gold (Bethesda  
Research Laboratories, catalog # 9532SA; Horisberger, M.  
(1981) Scanning Electron Microscopy 11:9) was used in the  
detection step described above instead of streptavidin  
30 tagged with the enzyme alkaline phosphatase. No further  
treatments were necessary and the cells were washed as  
described above. The presence of black precipitate or  
grains seen within cells when using either bright field or  
phase contrast microscopy techniques, or the visualization  
35 of bright white areas of light when using either

1 epi-polarization or dark field microscopy indicated that  
the cells contained target Diopolymer mRNA sequences  
substantially complementary to the probe, in this case to  
the c-abl gene.

5 EXAMPLE 10.

Detection of Nucleic Acids and Proteins in  
Peripheral Blood Cells.

10 Ten ml. of human peripheral blood were incubated  
at 37° C. in a 1.2% ammonium oxalate solution to lyse the  
red blood cells. The white blood cells were centrifuged  
at 3,000 rpm for 10 minutes in a clinical centrifuge. The  
cell pellet was subsequently washed with 10 ml. PBS and  
the pellet was resuspended in PBS. Cells were deposited  
15 by cytocentrifugation onto precleaned glass slides and air  
dried for 5 min. The cells were then fixed in 75%  
ethanol/20% acetic acid for 20 min. at room temperature.

No prehybridization step was performed. 20 ul of  
hybridization solution consisting of 50% formamide, 4x

20 SSC, 0.1M sodium phosphate (pH 7.4), 0.1% Triton X-100,  
100 ug/ml low molecular weight DNA (sheared herring sperm  
DNA obtained from Sigma Chemical Co.) and 2.5 ug/ml of  
c-abl anti-sense RNA probe labelled with Photobiotin <sup>TM</sup>,  
was added to each specimen. The anti-sense RNA probe was  
25 prepared as described in Example 1. After incubation for  
2 hrs. at 55°C, hybrid formation was detected.

To detect hybrids, streptavidin rhodamine complex  
at 2x the manufacturer's recommended concentration was  
added to the specimen. During this incubation, rabbit  
30 polyclonal antibody raised against the c-abl gene product  
(supplied by Dr. Russel Grieg of Smith Kline & French,  
Swedeland, Pennsylvania) was added to the specimen at a  
concentration which positively labeled K562 cells and did  
not show any detectable signal in HL60 cells when a  
35 fluorescein labeled anti-rabbit IgG was added.



1           After incubation at room temperature for 30  
minutes, the specimens were then gently washed (1-200 ml  
per cm<sup>2</sup> of cell area) with each of the following  
solutions containing 0.1% Triton-X 100, in order: 2x SSC,  
5   1x SSC, 0.5x SSC, and 0.1x SSC. One drop of a 50/50 (v/v)  
100% glycerol/2x PBS solution was added to each specimen.  
Specimens were photographed with high speed film (Kodak  
EES135, PS 800/1600) at 1600 ASA for 20 sec. exposure on a  
Nikon Photophot microscope at 400x magnification using a  
10   standard filter combination for transmission of  
fluorescent light.

          It is known that c-abl mRNA and protein are  
over-produced within the same cell in patients with  
chronic myelogenous leukemia (CML) (Stam, K. et al. N.  
15   Engl. J. Med. 313: 1429; Konopka, J.B. and Witte, O.N.  
(1984) Cell 37: 3116). Using the present invention,  
peripheral blood cells from a patient with CML were probed  
for the presence of mRNA corresponding the the c-abl gene  
and simultaneously, as described above, for the presence  
20   of the c-abl protein product. Figure 13 A demonstrates  
that the protein was readily detectable due to the  
fluorescein fluorescence emission of the reacted  
antibodies. In the same cells, Figure 13 B shows the  
presence of the c-abl mRNA, detectable due to the  
25   rhodamine fluorescence emission.

#### EXAMPLE 11.

##### Quantitation of the Number of Target Biopolymer Molecules.

          K562 Cells (ATCC #CCL 243) were grown in Hank's  
30   Balanced Salts Solution supplemented with 10% Fetal Calf  
Serum. Three days after the last change in media, the  
cells were split to a density of about 10<sup>5</sup> cells per 0.3  
ml. of fresh media. One hour later, 60 replica slides were  
made by depositing 50,000-100,000 cells onto a slide by  
35   cytocentrifugation. The remainder of the cells were

1 harvested and RNA and DNA was extracted from the cells by  
the guanidium cesium chloride method (Chirgwin, et al.  
(1979) Biochemistry 18: 5294).

5 Since the cell line was a relatively homogeneous  
population, the extracted RNA was purified and used to  
determine copy number estimates for each RNA species  
analyzed; i.e., an estimate could be made of the number of  
molecules of each gene present within each cell from a  
series of control experiments well known to those with  
10 knowledge and skill in the art. These control experiments  
to determine the number of molecules per cell included the  
following: Northern blots, RNA dot blots, Quick-blot<sup>TM</sup>,  
Cytodots<sup>TM</sup>, single copy saturation experiments, and  
solution concentration versus time hybridization  
15 experiments (Rot<sub>1/2</sub> analysis) (Hames, B.D. and Higgins,  
S.J. (1986) in Nucleic Acid Hybridization: a practical  
approach, IRL Press, Oxford-Washington, D.C.).

Cells on slides were fixed with 75% ethanol/20%  
glacial acetic acid/5% water for 20 minutes at room  
20 temperature.

No prehybridization step was performed. 20 ul of  
hybridization solution consisting of 50% formamide, 4x  
SSC, 0.1 M sodium phosphate (pH 7.4), 0.1% Triton X-100,  
100 ug/ml low molecular weight DNA (sheared herring sperm  
25 DNA obtained from Sigma Chemical Co.) and 2.5 ug/ml of an  
anti-sense RNA probe labelled with Photobiotin<sup>TM</sup>, was  
added to each specimen. Probes used were either the sense  
or anti-sense RNA strands the following genes: c-abl,  
c-sis, c-myc, or Epstein Barr Virus (EBV). The probes  
30 were prepared as described in Example 1. After incubation  
for 2 hours at 55° C, hybrid formation was detected.

To detect hybrids, streptavidin fluorescein  
(SA-FlTC), phycoerytherin (SA-PE), rhodamine B(SA-R),  
Texas Red<sup>TM</sup> (SA-TR), phycocyanin (SA-PC), or  
35 allophycocyanin (SA-APC) complexes were added at 2x the

1 manufacturer's recommended concentration. (SA-FITC,  
SA-TR: Bethesda Research Laboratories; SA-R: Southern  
Biologicals; SA-PE, SA-PC, SA-APC: BioMada). After  
incubation at 37° C for 10 minutes, the specimens were  
5 then gently washed with (1-200 ml. per cm<sup>2</sup> of cell area)  
with 0.1x SSC containing 0.1% Triton X-100. One drop of a  
50/50 (v/v) 100% glycerol/2x PBS solution was added to  
each specimen and a #1 coverslip was placed over the cells  
before microscopic examination.

10 Fluorescence emitted from each cell is a  
reflection of the number of streptavidin molecules which  
reacted with probe; the amount of reacted probe within a  
cell is indicative of the number of target biopolymers  
present within the cell. To measure the fluorescence  
15 within each cell, slides were analyzed using the ACAS 470  
Workstation from Meridian Instruments (Okemos, MI). The  
Meridian instrument, like most image processing systems,  
excites the fluors present within a sample and then  
captures the emitted light as either a digital or analog  
20 signal. This signal is digital on the Meridian  
instrument. The quantity of the signal can be represented  
by different colors. In Figure 14, this is illustrated in  
the top right hand panel which shows the colors the  
instrument assigns to emitted signals of different  
25 intensities. When these colors are represented over a cell  
(Figs. 14 A-C), the relative amount of emitted  
fluorescence per cell can be seen. In Figure 14A shows  
the detection of the c-sis gene; the intensity of emission  
of reacted fluorescer is seen; in Figure 14B, the  
30 detection of c-myc is shown. Figure 14C shows the  
background signal emitted when no probe is included in the  
hybridization solution. This panel is a negative control  
and is blank. The Meridian instrument can determine the  
total fluorescence over the entire cell (i.e., quantity of  
35 fluorescence per cell) and represent this information

graphically. The control experiments described above which were carried out with purified RNA from other cells showed that both the c-sis and c-myc cellular target genes were present in these cells at between 1 and 10 molecules per cell. Therefore, this value represents the scale on the horizontal axis in Figures 14D and 14E. The present invention together with the appropriate instrumentation, was capable of identifying the number of cells which contained even a single molecule of either the c-sis or c-myc gene.

#### EXAMPLE 12.

##### Error Rates of the In Situ Hybridization System.

K562 Cells (ATCC #CCL 243) were grown in Hank's Balanced Salts Solution supplemented with 10% fetal calf serum. Three days after the last change in media, the cells were split to a density of about  $10^5$  cells per 0.3 ml of fresh media. One hour later, 60 replica slides were made by depositing 50,000-100,000 cells onto a slide by cytocentrifugation. The remainder of the cells were harvested and RNA and DNA was extracted from the cells by the guanidium cesium chloride method as in the previous Example 11.

Since the cell line was a relatively homogeneous population, the extracted RNA was purified and used to determine copy number estimates for each RNA species analyzed; i.e., an estimate could be made of the number of molecules of each gene present within each cell from a series of control experiments well known to those with knowledge and skill in the art. These control experiments to determine the number of molecules per cell included the following: Northern blots, RNA dot blots, Quick-blots™, Cytodots™, single copy saturation experiments, and solution concentration versus time hybridization experiments (Rot<sub>1/2</sub> analysis) (Hames, B.D. and Higgins,

1 S.J. (1986) in Nucleic Acid Hybridization: a practical approach. IRL Press, Oxford-Washington, D.C.).

Cells on slides were fixed with 75% ethanol/20% glacial acetic acid/5% water for 20 minutes at room temperature.

5 No prehybridization step was performed. 20 ul of hybridization solution consisting of 50% formamide, 4x SSC, 0.1 M sodium phosphate (pH 7.4), 0.1% Triton X-100, 100 ug/ml low molecular weight DNA (sheared herring sperm DNA obtained from Sigma Chemical Co.) and 2.5 ug/ml of an anti-sense RNA probe labeled with Photobiotin™, was added to each specimen. Probes used were either the sense or anti-sense RNA strands of the following genes: c-abl, c-sis, c-myc, or Epstein Barr Virus (EBV). The probes were prepared as described in Example 1. After incubation for 2 hours at 55° C, hybrid formation was detected.

15 To detect hybrids, streptavidin fluorescein (SA-FlTC), phycoerytherin (SA-PE), rhodamine B(SA-R), Texas Red™ (SA-TR), phycocyanin (SA-PC), allophycocyanin (SA-APC) complexes were added at 2x the manufacturer's recommended concentration. (SA-FlTC, SA-TR: Bethesda Research Laboratories; SA-R: Southern Biologicals; SA-PE, SA-AP: BioMead). After incubation at 37° C for 10 minutes, the specimens were then gently washed (1-200 ml per cm<sup>2</sup> of cell area) with 0.1x SSC containing 0.1% Triton X-100™. One drop of a 50/50 (v/v) 100% glycerol/2x PBS solution was added to each specimen and a #1 coverslip was placed over the cells before microscopic examination.

30 Fluorescence emitted from each cell is a reflection of the number of streptavidin molecules which reacted with probe; the amount of reacted probe within a cell is indicative of the number of target biopolymers present within the cell. To measure the fluorescence within each cell, slides were analyzed using the ACAS 470

35

1        Workstation from Meridian Instruments (Okemos, MI). The  
Meridian instrument, like most image processing systems,  
excites the fluors present within a sample and then  
captures the emitted light as either a digital or analog  
5        signal. This signal is digital on the Meridian  
instrument. In a manner similar to the method described  
in Example 11, the total fluorescence per cell was  
determined using the ACAS 470 workstation. The data  
obtained was then analyzed by the Mann-Whitney test to  
10        determine if there were statistically significant  
differences between the amounts of fluorescence seen when  
different probes were used in the in situ hybridization  
system. In a cell line which has a known target  
biopolymer RNA present, a probe should react with the  
15        target; this would lead to the generation of a fluorescent  
signal within the positive cells. In cases in which the  
"target" biopolymer RNA is known to be absent from the  
cells, a probe reactive to the target should not bind in  
any non-specific manner to the cells and thus should not  
20        generate any fluorescent signal within the cells. A  
statistical test can determine whether this is true and  
whether the difference between the "positive" and the  
"negative" is sufficiently different to be correct and not  
random. Furthermore, the statistical test can determine  
25        the probability of the test incorrectly identifying a  
negative sample as positive or a positive sample as  
negative. Table 1 shows the results of this statistical  
analysis. The positive samples were correctly  
identified. The error rates represent the chance of  
30        obtaining false results when different thresholds of  
sensitivity for the present invention are employed.

TABLE 1

In Situ Hybridization  
False Positive, False Negative Rates

<u>Detection Threshold</u>	<u>Error Rate</u>
1-2 genes/cell	1.71%
1-5 genes/cell	0.65%
>10 genes/cell	>0.005%

EXAMPLE 13.Detection of Cytomegalovirus in Peripheral Blood.

One ml. of human peripheral blood was obtained from the patients described in Example 8 and processed as described in that Example. The hybridization reaction was carried out with the specimens using the same hybridization cocktail described in Example 4, except the probe was an anti-sense RNA probe complementary to cytomegalovirus (CMV) RNA and labeled with Photobiotin™. Hybrid detection was carried out as described in Example 8.

The column of photomicrographs in Figures 15 and 16 on the left (CMV) illustrates that the present invention is capable of detecting target viral biopolymers --- here detecting CMV --- within a specimen. The presence of CMV in the specimen is indicated by the emitted light within the cells. The column of photomicrographs in Figures 15 and 16 on the right (BLANK) shows no emitted light; these pictures show that in the controls no extraneous signals were produced.

EXAMPLE 14.Detection of HIV in Peripheral Blood Cells  
of an Individual Seronegative for HIV  
But at High Risk for HIV Infection.

Ten ml. of human peripheral blood from an individual at high risk for HIV infection was obtained, processed, hybridized and hybrids detected and photographed as described in Example 8. In Figure 17, the panels marked GAG, ENV, TAT, LTR, EBV represent results obtained when the corresponding anti-sense RNA probes were added to the hybrid solution. The panels with HIV anti-sense probes added are positive while EBV is negative. The panel marked "Blank" represents results obtained when no probe was added to the hybrid solution and is negative. The bottom right panel is a phase contrast photomicrograph of the cells in the panel marked "Blank".

To confirm that HIV was present in the blood cells of this individual, a Southern blot analysis (Southern, (1975) J. Mol. Biol. 98:503) of DNA from the HIV infected cell line H-9 (ATCC #CRL 8543) (lanes A and C) and from the peripheral blood cells from this same seronegative but high risk individual (lanes B and D) is presented in Figure 19. DNA in lanes A and B was digested with Sst I and in lanes C and D with Hind III. The blot was hybridized with a full length HIV probe, radiolabelled with  $^{32}\text{P}$ , and demonstrates that HIV hybridizing sequences are present in the peripheral blood cells of this individual.

EXAMPLE 15.Usefulness of In Situ Hybridization  
to Monitor Effectiveness of Patient Therapy.

Peripheral blood was obtained from patients with chronic myelogenous leukemia (CML) both before and after



1 treatment with either alpha or gamma interferon. The  
blood was processed, hybridization was accomplished, and  
hybrids were detected as described in Example 6. Figure  
19 demonstrates that in a CML patient before  
5 alpha-interferon treatment (Day 0) the c-myc, c-sis, and  
c-abl oncogene target biopolymers were all present, as  
demonstrated by the light emitted from the cells and seen  
on the photomicrographs at day 0. In the same patient,  
the same target cellular genes were not produced after  
10 four days of alpha-interferon therapy (little or no signal  
is seen in the cells at day 4). In contrast, in a patient  
who underwent treatment with gamma-interferon, cells were  
still present which over-produced the c-sis and c-abl  
oncogene (Figure 20, Panels E and F). Clinically, the  
15 patient who was treated with alpha-interferon responded  
well to the therapy and went into remission. The patient  
who received gamma-interferon failed to respond to this  
therapy. The monitoring of changes in the type or amount  
of a cellular target biopolymer sequence may be an  
20 important means of evaluating or predicting the  
effectiveness of therapeutics.

One skilled in the art will readily appreciate  
that the present invention is well adapted to carry out  
the objects and obtain the ends and advantages mentioned,  
25 as well as those inherent therein. The components,  
methods, procedures and techniques described herein are  
presently representative of the preferred embodiments, are  
intended to be exemplary, and are not intended as  
limitations on the scope of the present invention.  
30 Changes therein and other uses will occur to those skilled  
in the art which are encompassed within the spirit of the  
invention and are defined by the scope of the appended  
claims.

35 What is claimed is:

1           1.    A method for assaying biopolymers in a  
specimen having substantially intact membranes comprising  
the steps of:

5                contacting said specimen with a fixation  
medium comprising at least one agent selected  
from the group consisting of a precipitating  
agent and a cross linking agent,

10               contacting said fixed specimen with a  
hybridization solution consisting of a denaturing  
agent, a hybrid stabilizing agent, a buffering  
agent, a selective membrane pore-forming agent  
and at least one probe having a nucleotide  
15               sequence at least substantially complementary to  
a specific target nucleotide sequence to be  
detected, said contacting being under hybridizing  
conditions at a temperature of 15-80°C for about  
20-120 min.,

              incubating said specimen with said medium in  
the presence of at least one detectable label,

20               detecting duplex formation by means of said  
label, wherein said method is capable of  
detecting as few as 1-5 biopolymers per cell.

25           2.    The method of Claim 1 wherein said label is  
attached to said probe.

              3.    The method of Claim 1 wherein said label is  
added after the duplex formation is complete.

30           4.    The method of Claim 1 wherein said label is  
selected from the group consisting of radioactive labels,  
fluorescers, chemiluminescers, enzyme labels, and  
radiolabels.

1           5.    The method of Claim 3 wherein said label is  
selected from the group consisting of avidin and  
streptavidin.

5           6.    The method of Claim 1 wherein said  
precipitating agent is selected from the group consisting  
of ethanol, methanol, acetone, formaldehyde and  
combinations thereof.

10          7.    The method of Claim 1 wherein said  
cross-linking agent is selected from the group consisting  
of paraformaldehyde, formaldehyde, dimethylsilserimide,  
and ethyldimethylamino-propylcarbodiimide.

15          8.    The method of Claim 1 wherein said  
denaturing agent is selected from the group consisting of  
formamide, urea, sodium iodide, thiocyanate, guanidine,  
perchlorate, trichloroacetate, and tetramethylamine.

20          9.    The method of Claim 1 wherein said hybrid  
stabilizing agent is selected from the group consisting of  
sodium chloride, lithium chloride, magnesium chloride, and  
ferric sulfate.

25          10.   The method of Claim 1 wherein said pore  
forming agent is selected from the group consisting of  
Brij 35, Brij 58, Triton X-100, CHAPS<sup>TM</sup>, desoxycholate  
and dodecyl sulfate.

30          11.   The method of Claim 1 wherein said  
biopolymer is RNA.

35          12.   The method of Claim 1 wherein said  
biopolymer is DNA.

1           13. The method of Claim 1 wherein said  
biopolymer is an antigen.

5           14. The method of Claim 1 wherein at least two  
biopolymers are assayed simultaneously in the same  
sample.

10           15. The method of Claim 14 wherein at least one  
biopolymer is a polynucleotide and a second biopolymer is  
an antigen.

16. The method of Claim 1 wherein said  
temperature is 15°C- 80°C.

15           17. The method of Claim 16 wherein said  
temperature is 50°C to 55°C.

18. The method of Claim 1 wherein said method is  
accomplished within about 4 hours.

20           19. The method of Claim 1 wherein said  
biopolymer is selected from the group consisting of a RNA,  
a DNA, a viral gene, an oncogene, and an antigen.

25           20. A method of assaying biopolymers in  
peripheral blood and bone marrow cells having  
substantially intact membranes comprising the steps of:  
          depositing said specimen on a solid support,  
          contacting said specimen with a fixation  
30           medium comprising 75% ethanol/20% glacial acetic  
acid/5% water for at least 10 minutes at a  
temperature ranging from -20°C to 50°C,  
          contacting said fixed specimen with a  
hybridization solution at about 50°-55°C for at  
35           least 5 min said hybridization solution

1 comprising about 20-80% formamide, about 5 times  
concentrated SSC, about 0.1M TRIS-HCl, pH 7.4,  
about 0.1% Triton X-100, and a photobiotinylated  
single stranded anti-sense RNA probe having  
5 75-150 bases at least substantially complementary  
to a specific target nucleotide sequence to be  
detected,

adding a detectably labeled agent selected  
from the group consisting of avidin and  
streptavidin at a concentration sufficient to  
10 bind said hybridized probe within at least 5 min,

washing said labeled specimen with 0.1x SSC  
containing 0.1% Triton X-100 to remove unbound  
labeled agent, and

15 detecting said bound label.

21. The method of claim 20, wherein said  
biopolymer is an oncogene.

22. The method of claim 20, wherein said  
20 biopolymer is a virus.

23. A method of assaying biopolymers in tissue  
samples having substantially intact membranes comprising  
the steps of:  
25

depositing said tissue specimen on a solid  
support,

30 contacting said specimen with a fixation  
medium comprising 50% methanol/50% acetone for at  
least 20 minutes at a temperature ranging from  
-20°C to 50°C,

35 contacting said fixed specimen with a  
hybridization solution at about 50°-55°C for at  
least 5 min said hybridization solution

1 comprising about 20-80% formamide, about 5 times  
concentrated SSC, about 0.1M sodium phosphate,  
pH 7.4, about 0.1% Triton X-100, 20 mM vanadyl  
5 ribonucleoside complexes, low molecular weight  
denatured DNA at a concentration 100 times  
greater than the probe concentration and a  
photobiotinylated single stranded anti-sense RNA  
probe having 75-150 bases at least substantially  
10 complementary to a specific target nucleotide  
sequence to be detected,

adding a detectably labeled agent selected  
from the group consisting of avidin and  
streptavidin at a concentration sufficient to  
bind said hybridized probe within at least 5 min,

15 washing said labeled specimen with 0.1 X SSC  
containing 0.1% Triton X-100 to remove unbound  
labeled agent, and

detecting said bound label.

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1/20

Temperature Effect on In Situ  
Hybridizations

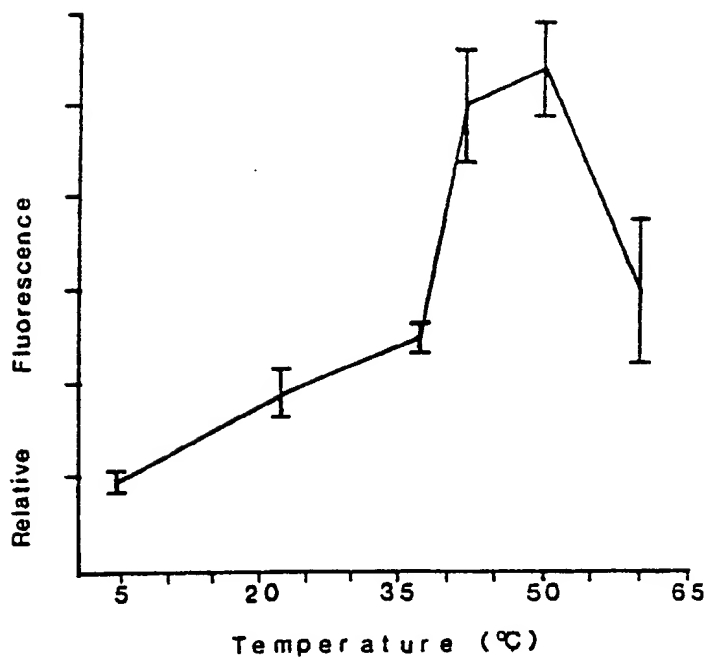


Figure 1

### In Situ Hybridization Kinetics

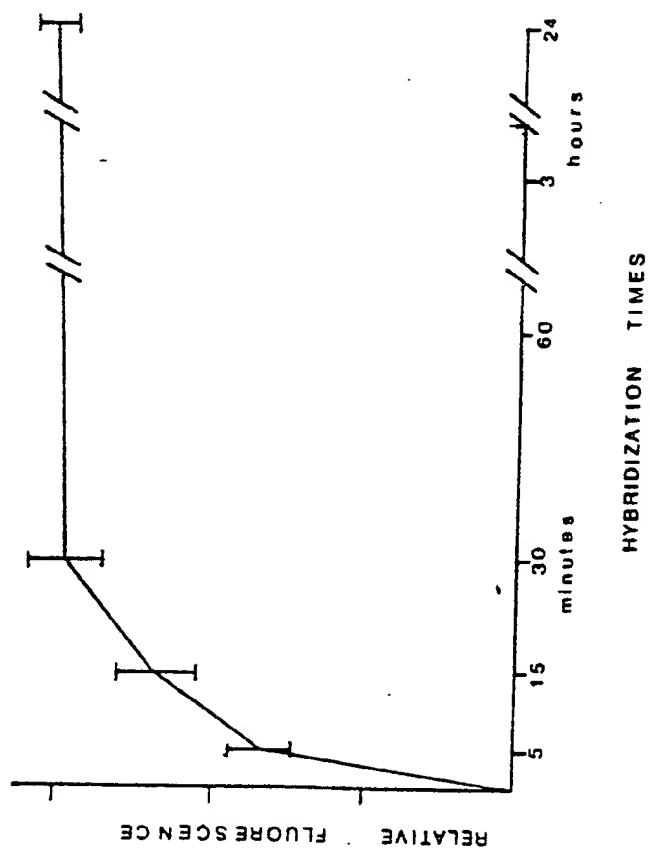


Figure 2



3/20

# SECONDARY STRUCTURE OF CELLULAR RNA

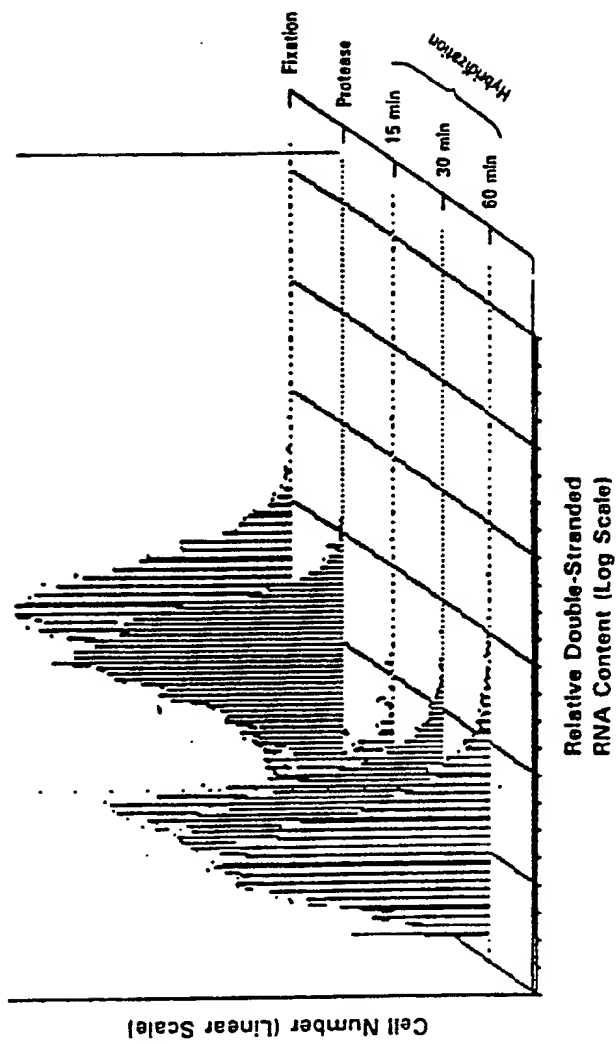


Figure 3

4/2a

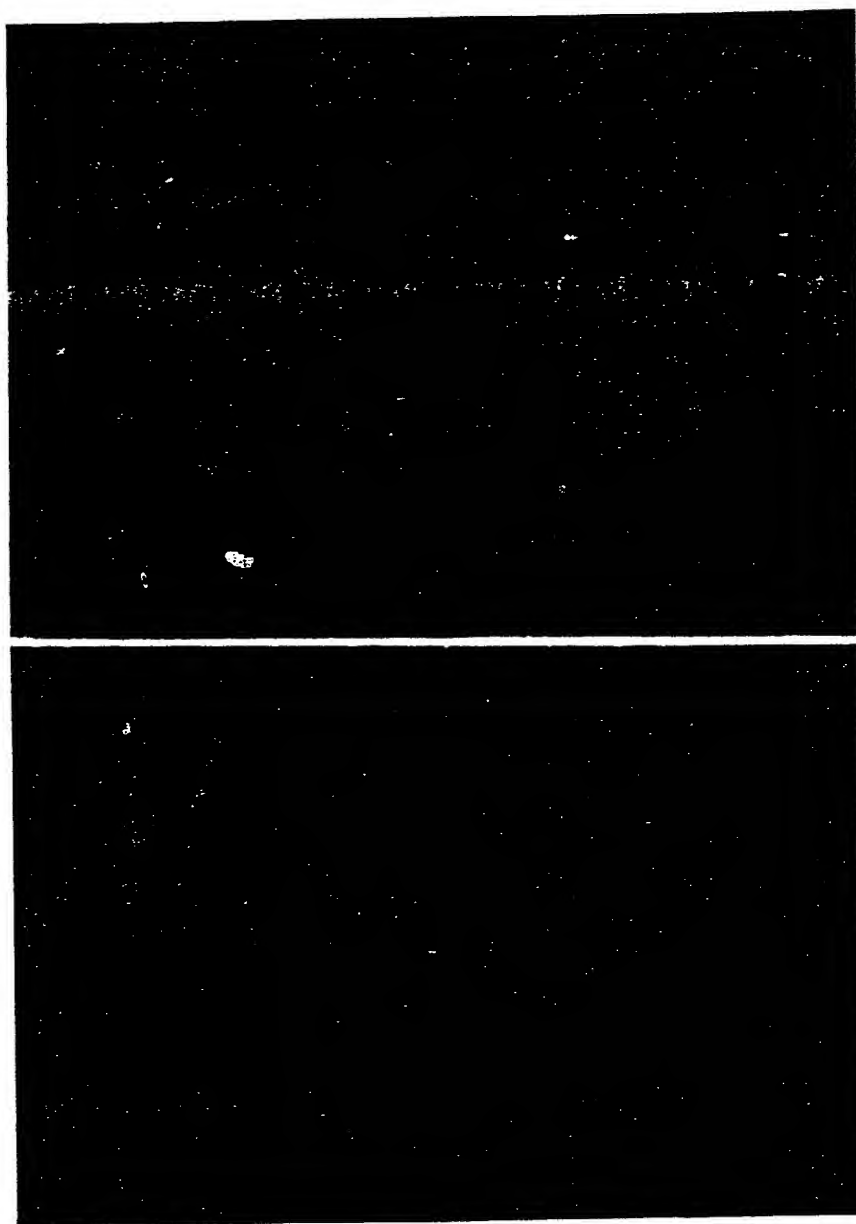
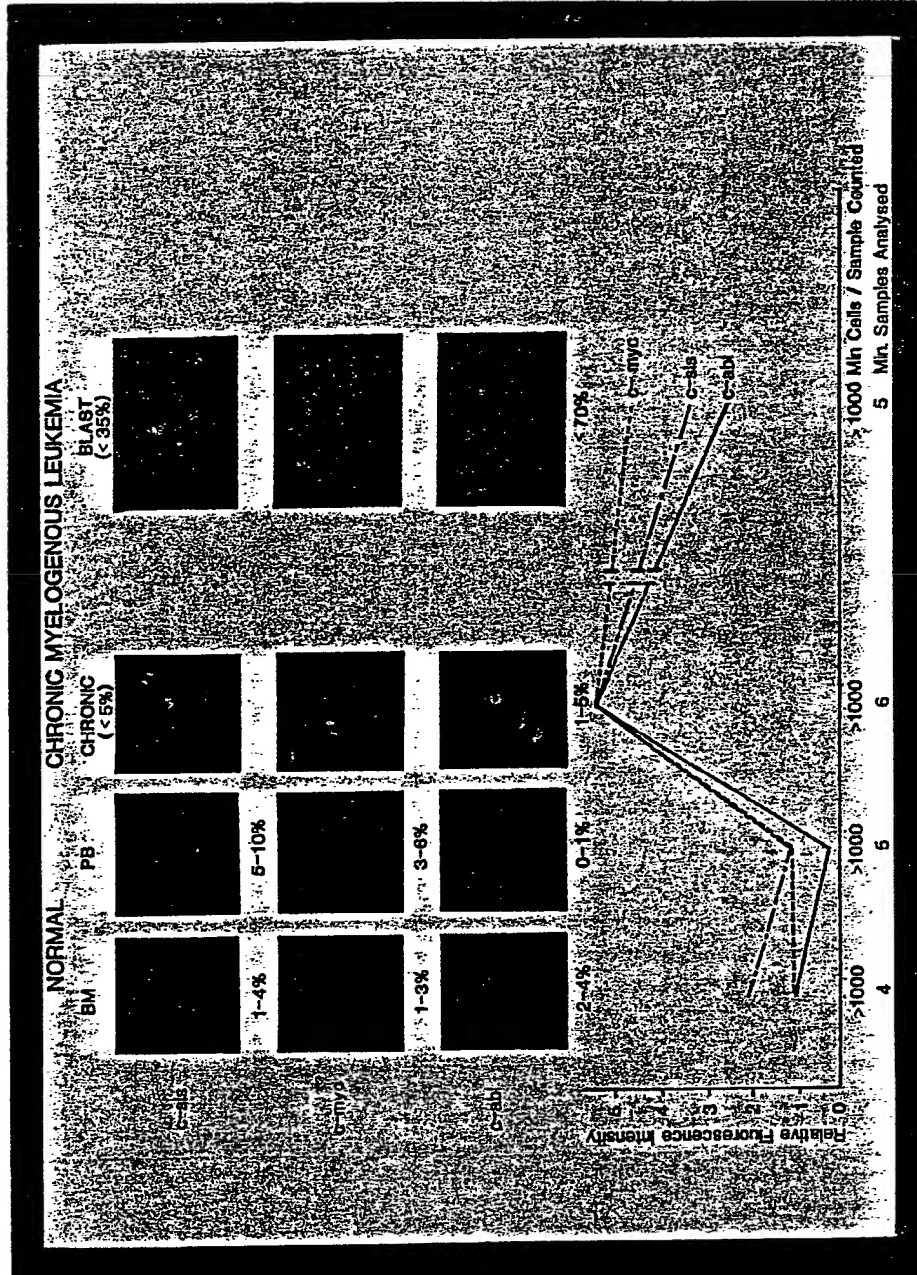


FIG. 4

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5/20



SUBSTITUTE SHEET

6/20

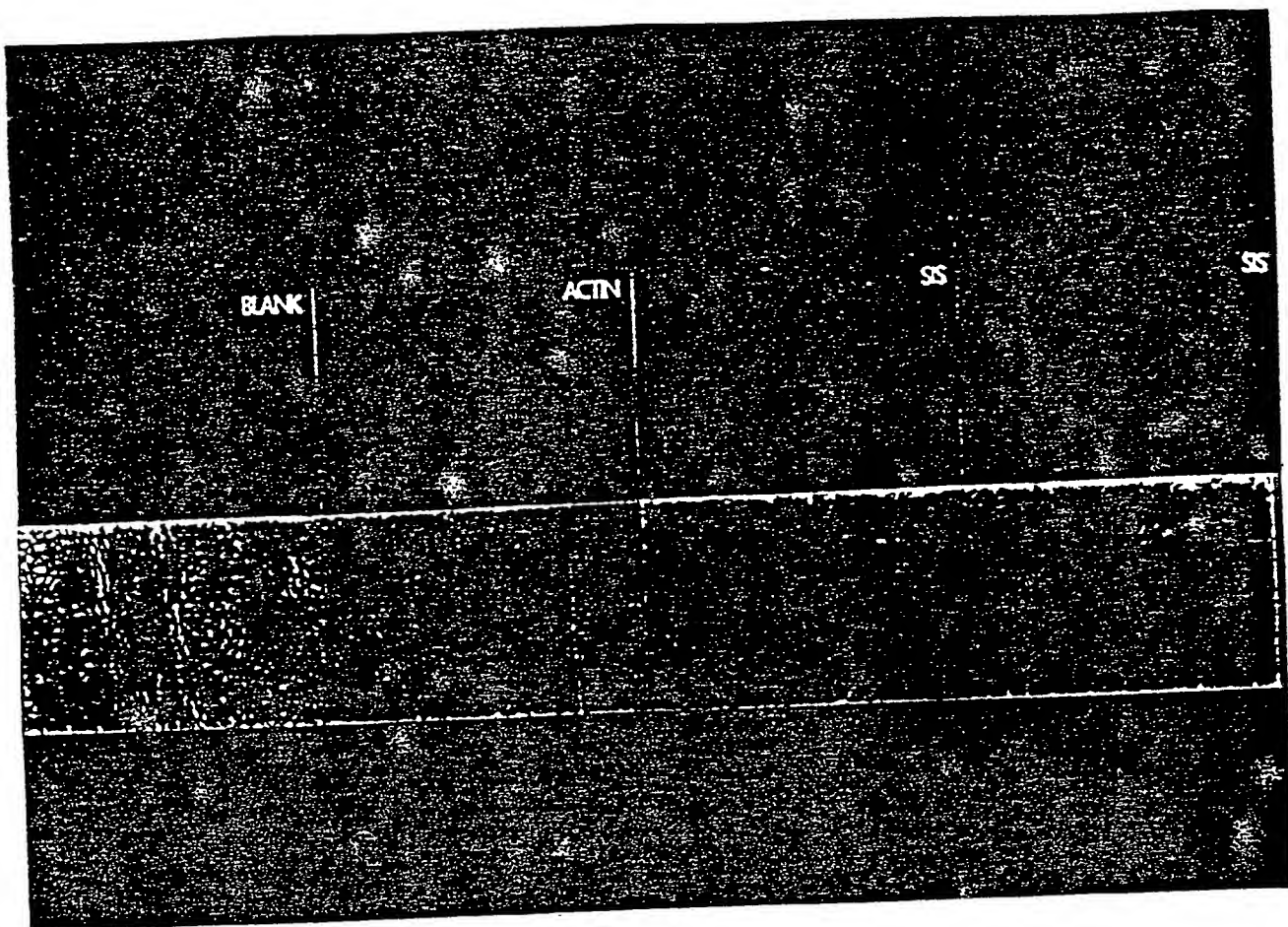


FIGURE 6

7/20

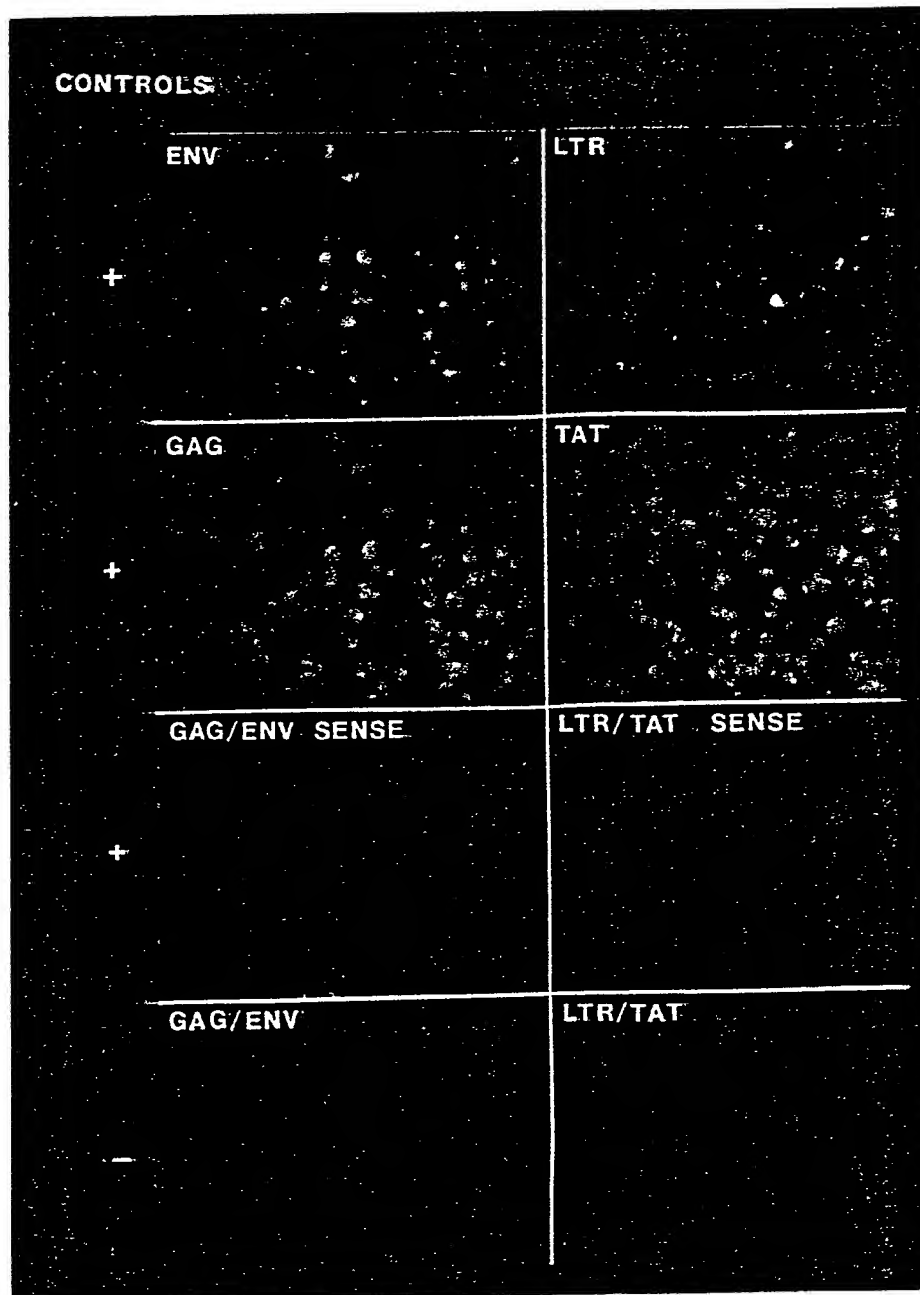


FIG. 7

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8/20

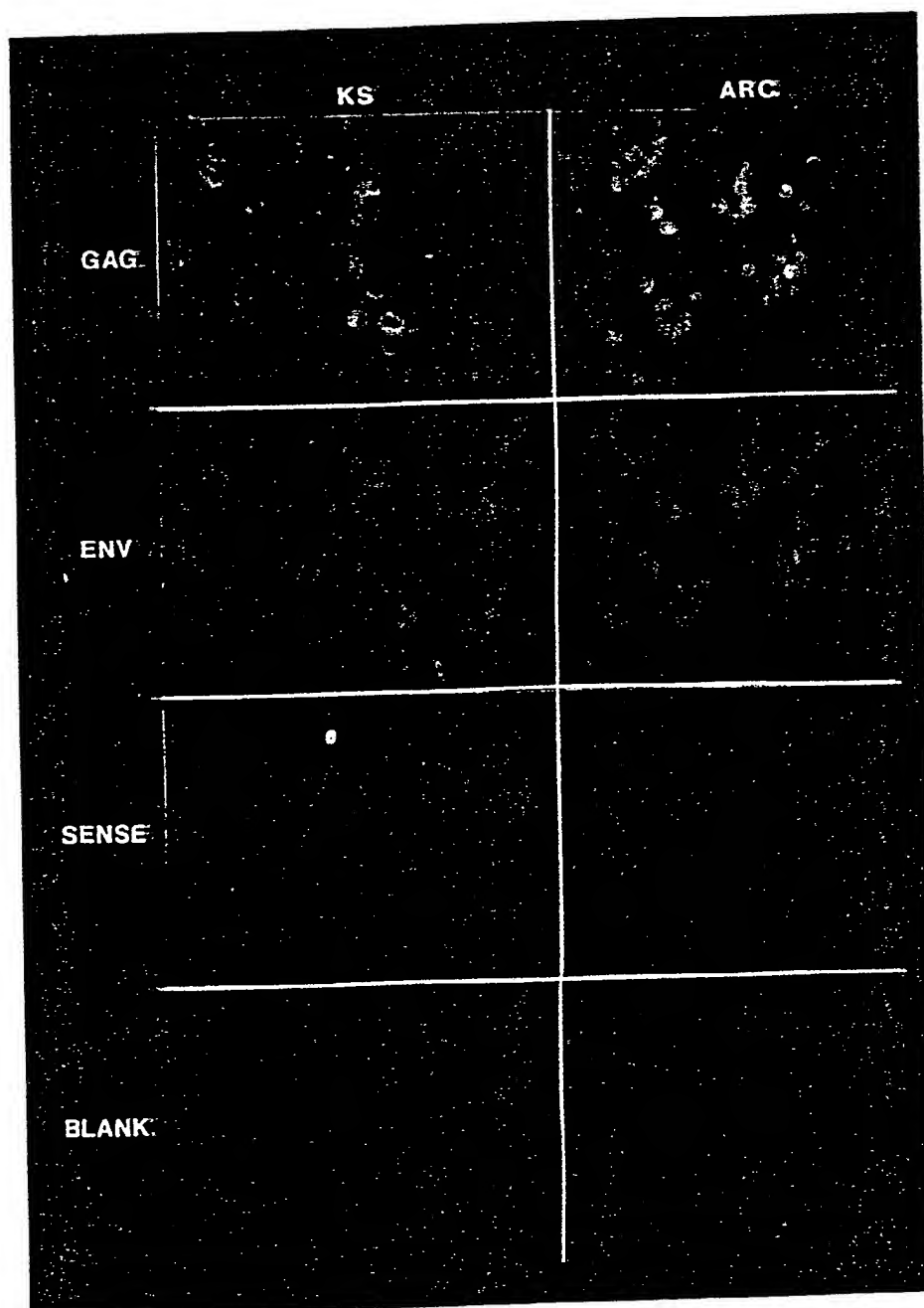


FIG. 8

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9/20

	AIDS	LYMPHOMA
GAG		
ENV		
SENSE		
BLANK		

FIG. 9

SUBSTITUTE SHEET

10/20

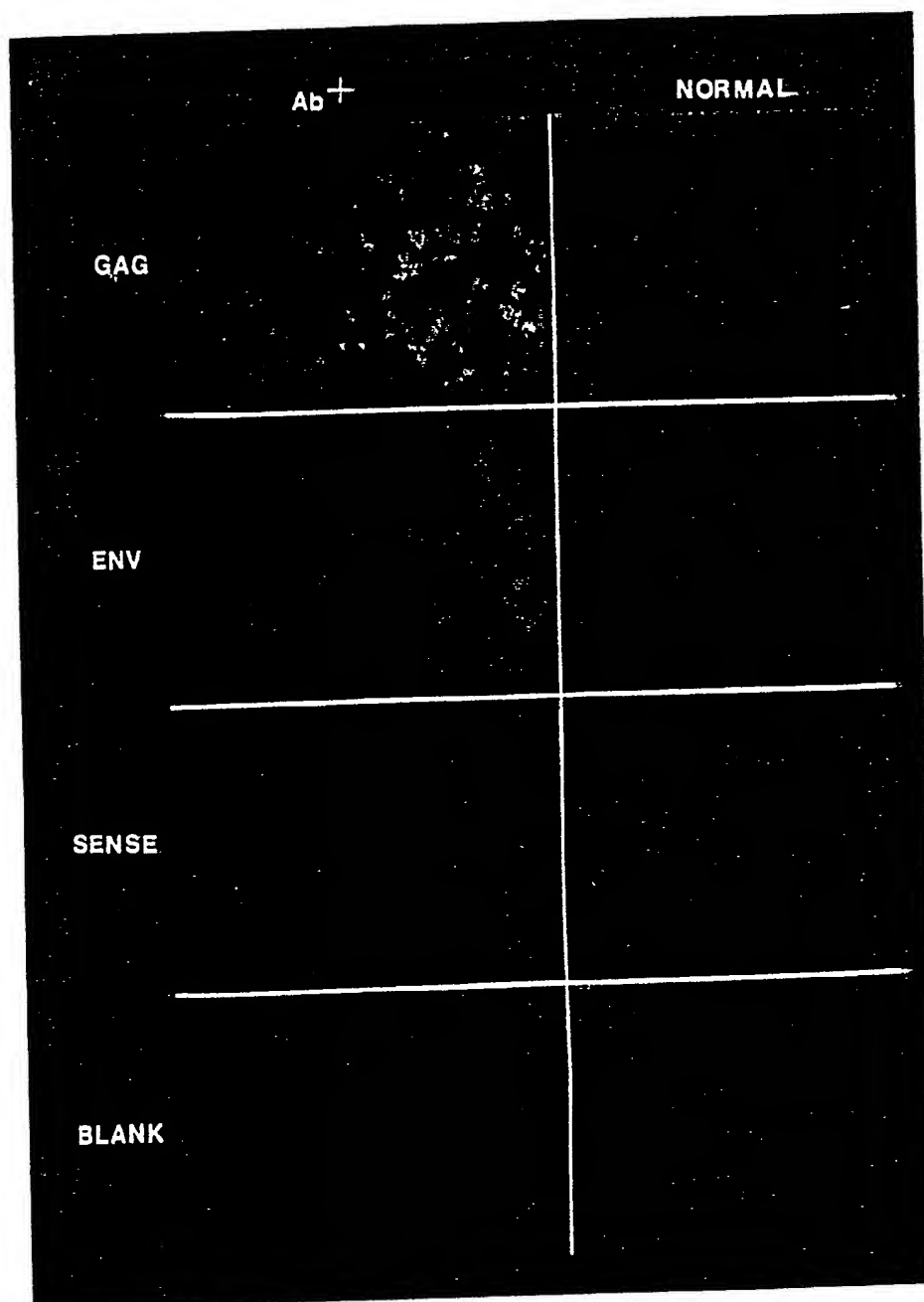


FIG. 10

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11/20

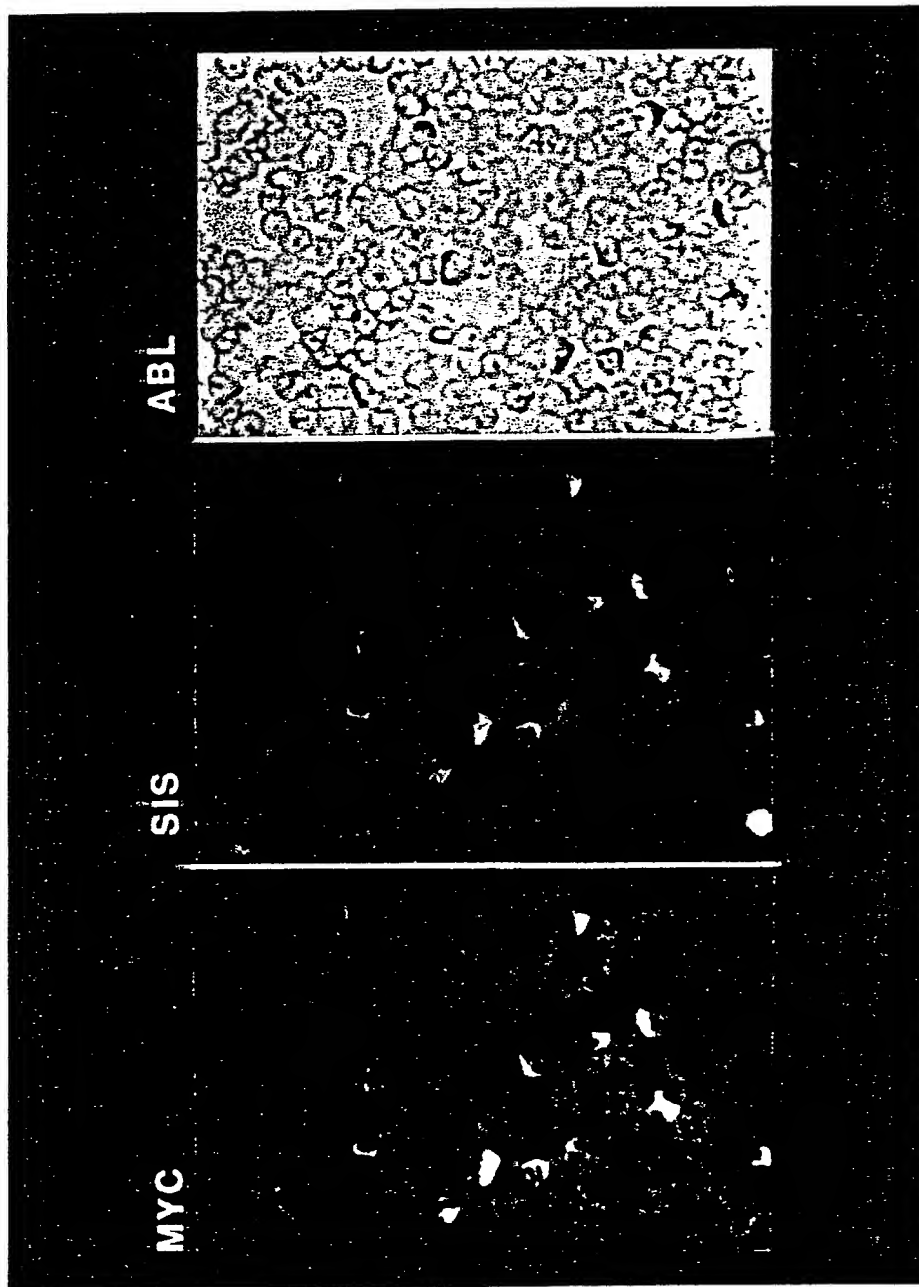


FIG. 11

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12/20

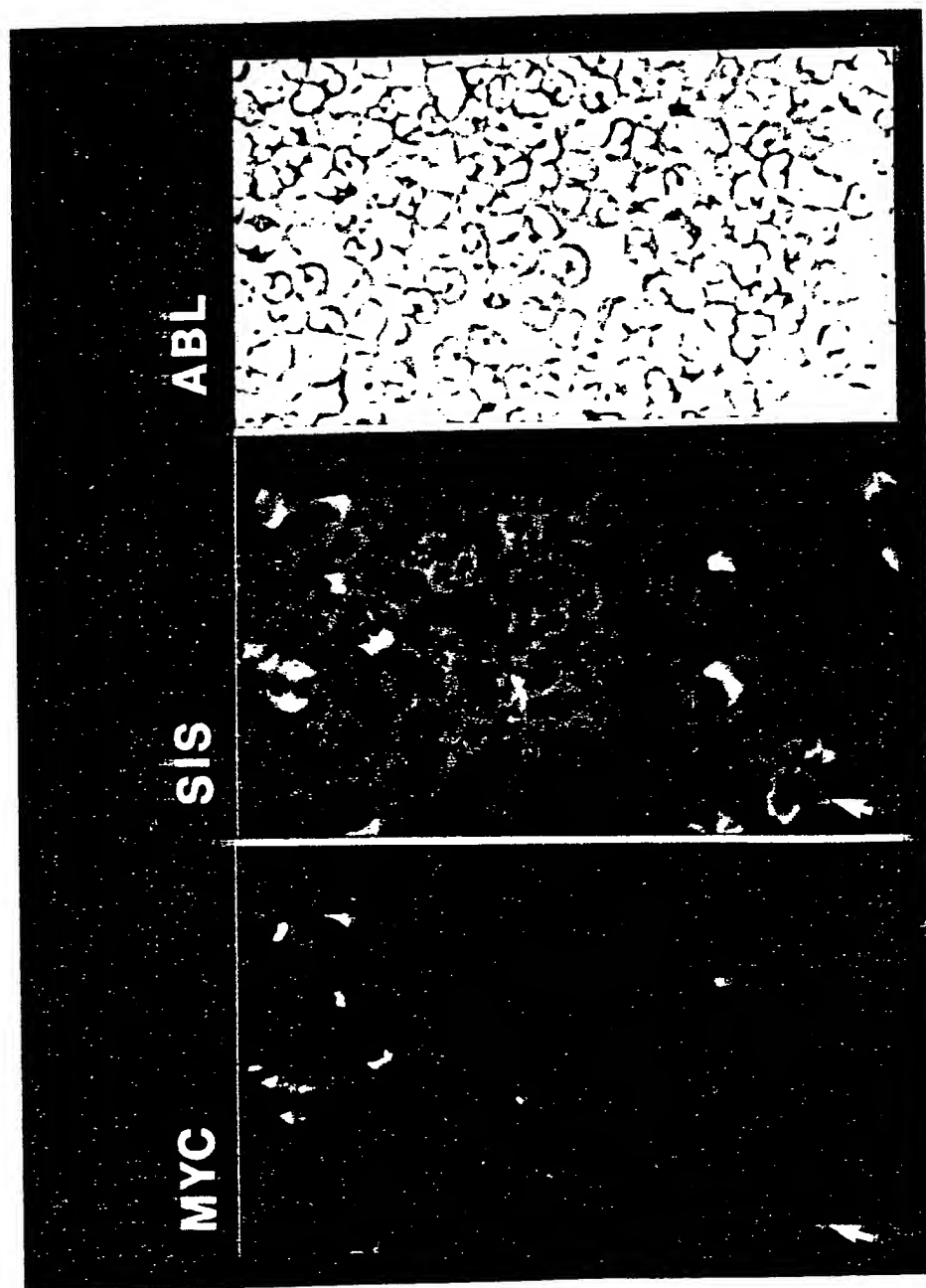


FIG. 12

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13/20

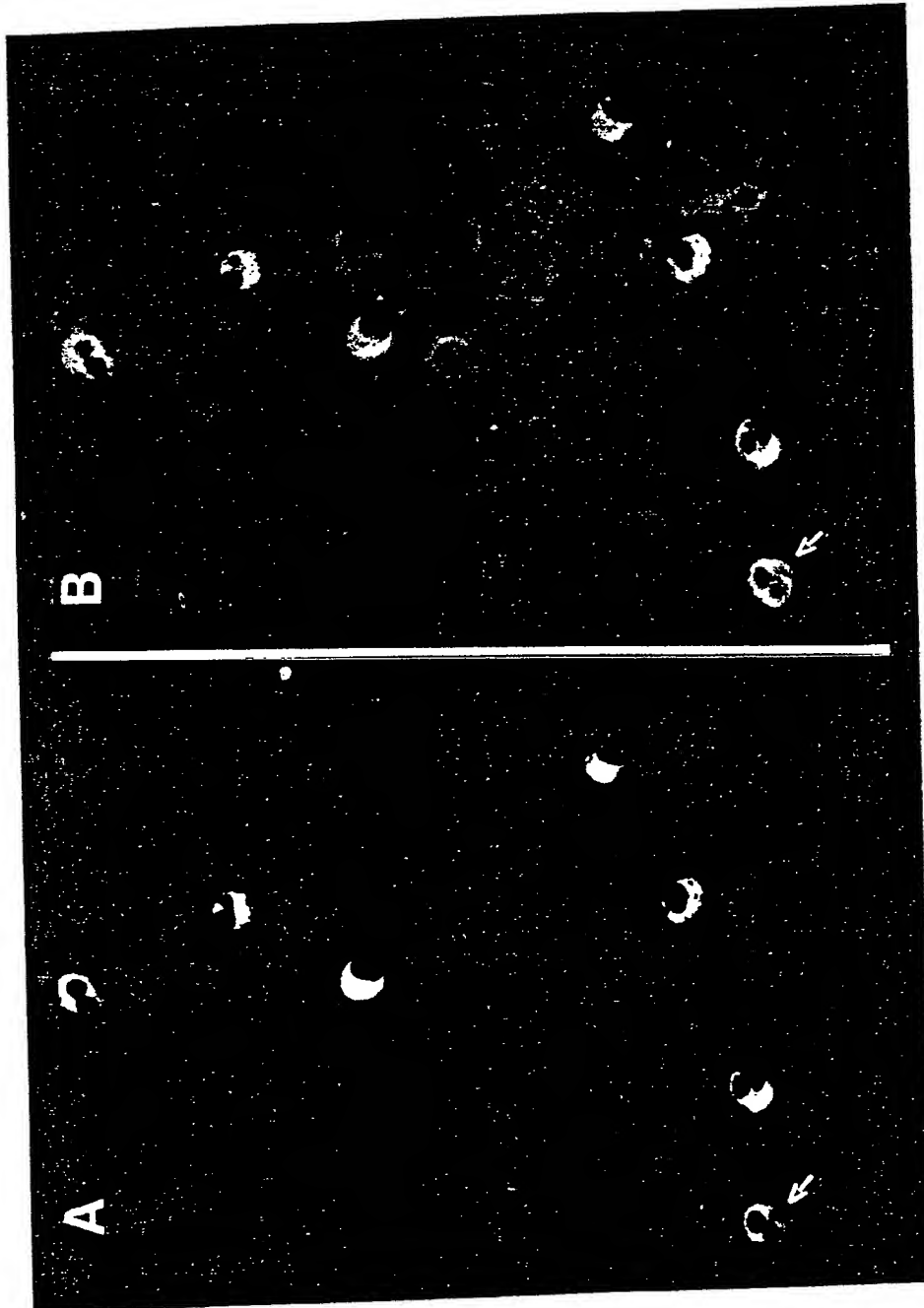


FIG. 13

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14/20

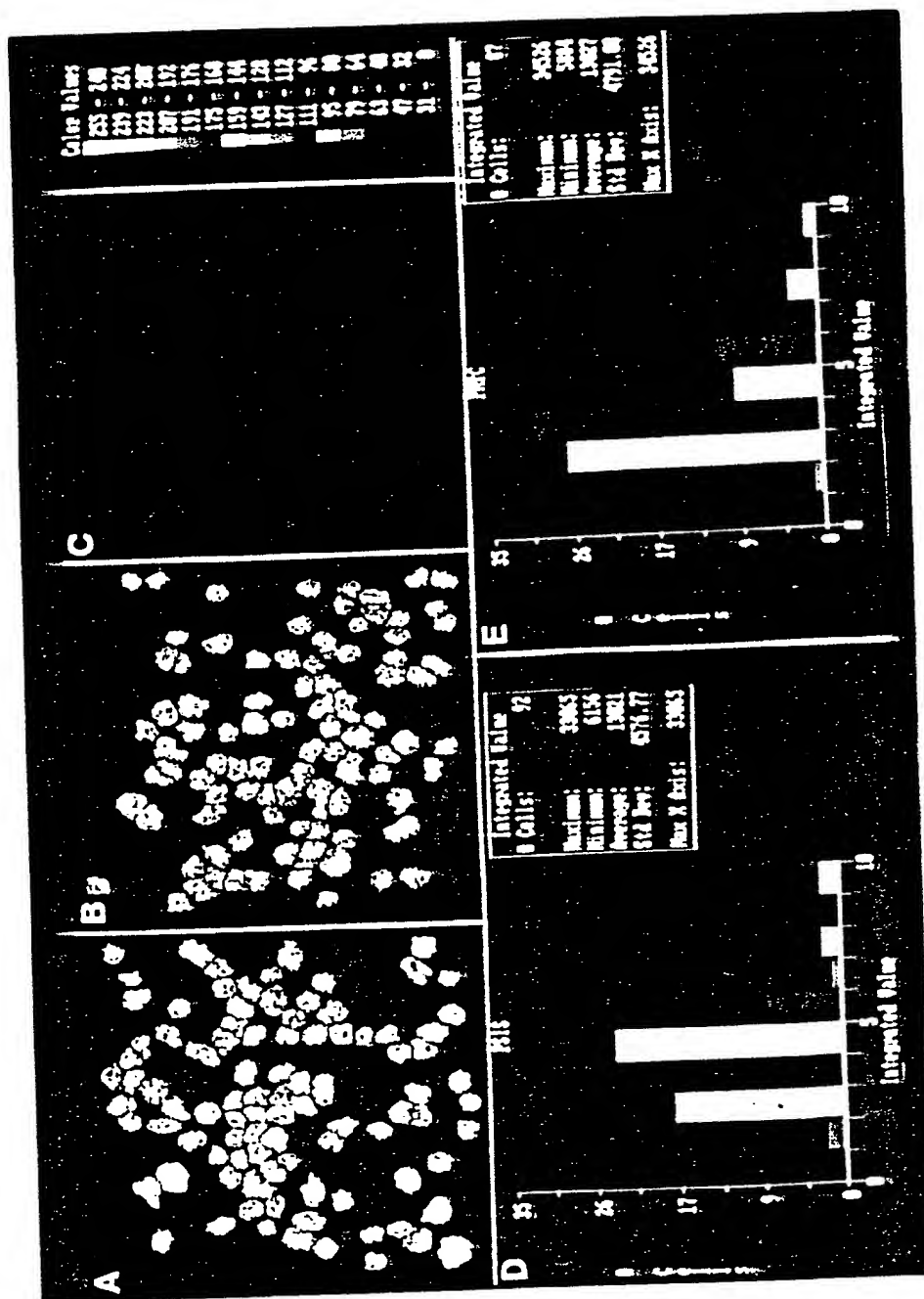


FIG. 14

15/20

	CMV	BLANK
KS		
ARC		
AIDS		
LYMPH		

FIG. 15

16/20

	CMV	BLANK
AB <sup>+</sup>		
N		
-N-		
-N-		

FIG. 16

SUBSTITUTE SHEET

17/20

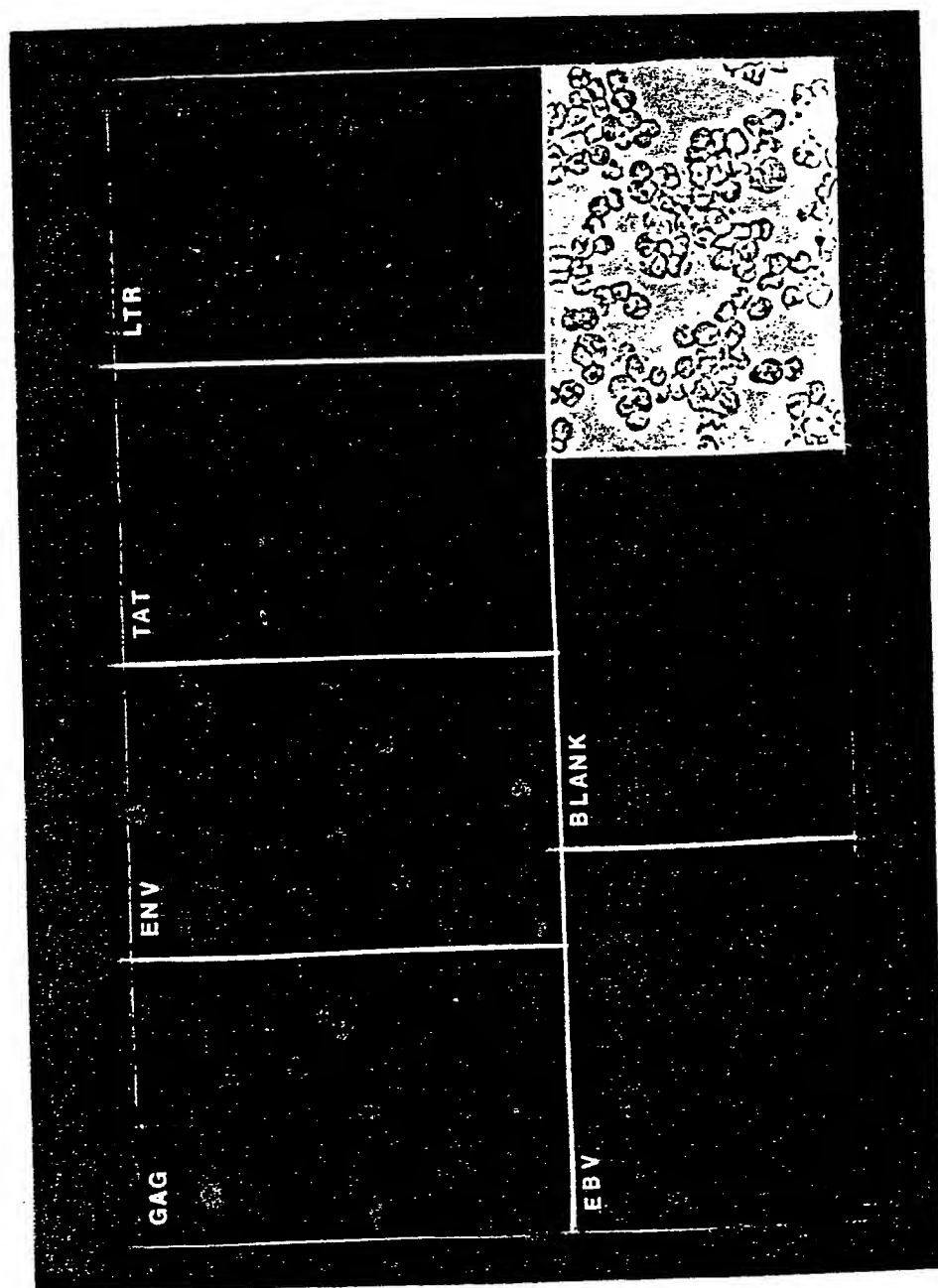


FIG.17

18/20

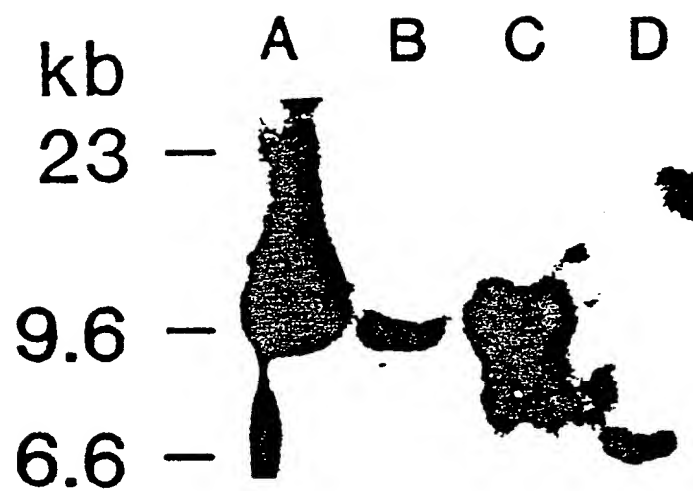


FIGURE 18



19/20

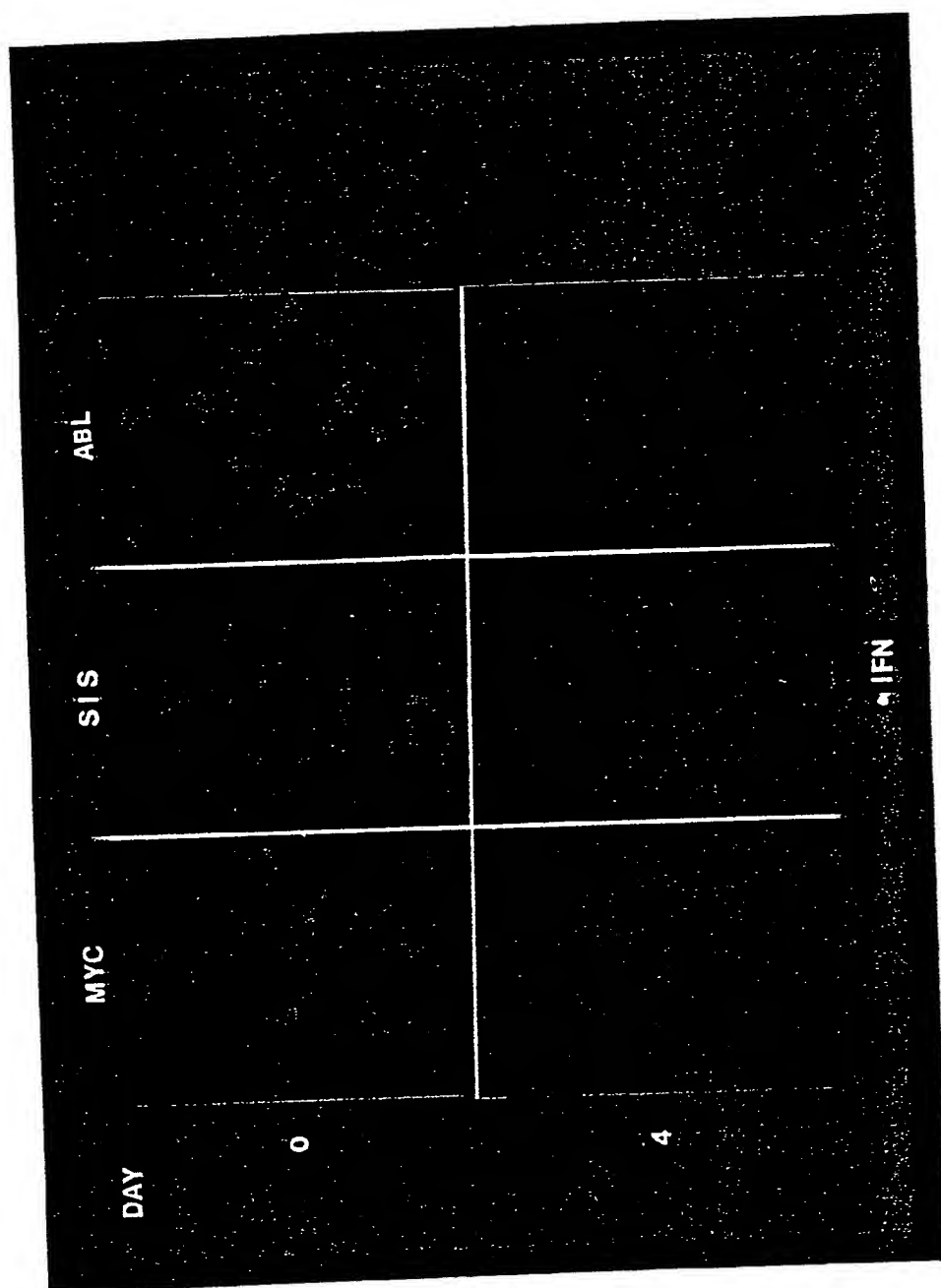


FIG. 19

20/20

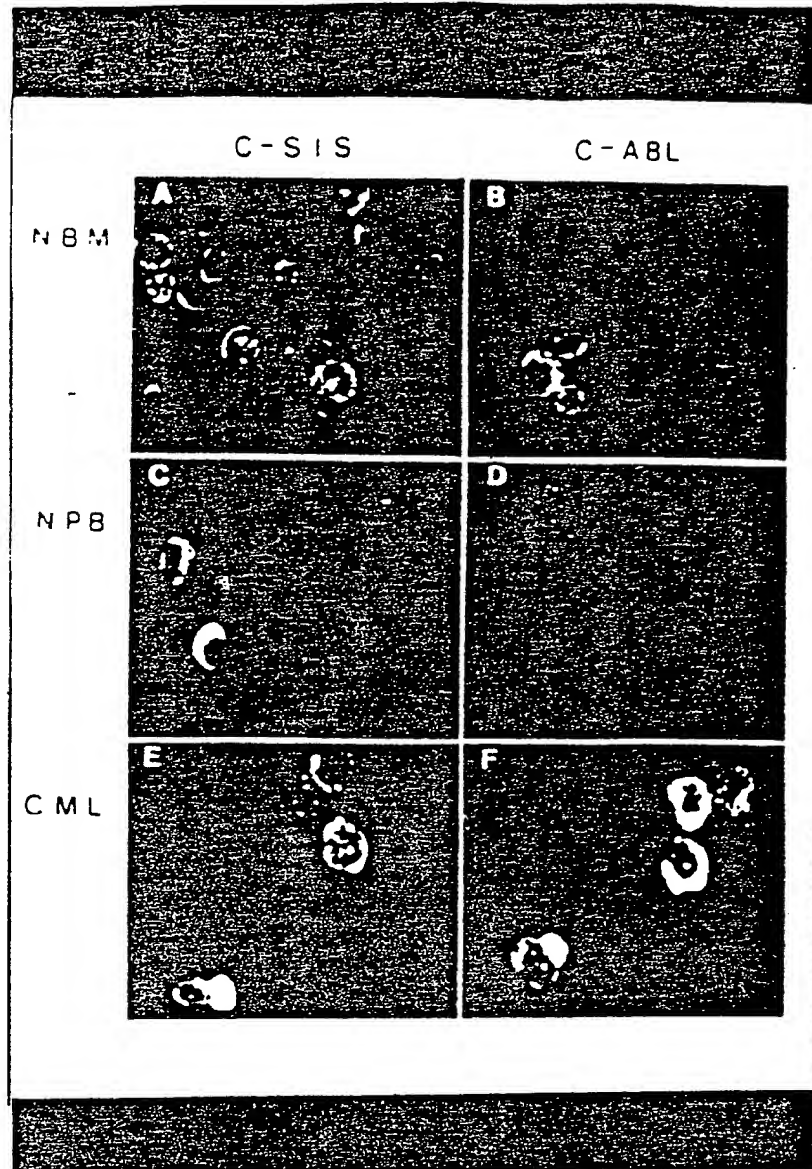


FIGURE 20

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03582

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC  
 IPC(4): C12Q 1/68; G01N 33/53  
 U.S. Cl.: 435/6, 7

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>7</sup>

Classification System

Classification Symbols

U.S.

435/6, 7

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>

Computer Search: Cas, Biosis; APS Data bases

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	WO, A, 86/04146 (United States of America) 17 July 1986.	1-23
Y	WO, A, 85/04720 (Howard Florey Institute of experimental Physiology and Medicine) 24 October 1985	1-23
Y	Laboratory Investigation, Volume 56 (1987) page 88A Wolber: "Cytomegalovirus Detection by <u>In-situ</u> , DNA Hybridization"	1-23
Y	Chemical Abstracts, Volume 107 number 19 issued 1987 (Columbus, Ohio USA) Bresser et al. "Comparison and optimization of <u>in situ</u> hybridization procedure yielding rapid, sensitive mRNA. detections" abstract No. 171731w, Gene Anal Tech 4(5) 89-104 1987	1-23

<sup>10</sup> Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

01 November 1989

**08 DEC 1989**

International Searching Authority

Signature of Authorized Officer

ISA/US

Scott A. Chambers

**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**

**Y**

Journal of Clinical Microbiology, Vol 22  
No. 4 issued October 1985 (Washington D.  
C. USA) Forghani et al. "Rapid detection  
of Herpes Simlex Virus DNA in human  
Brain Tissue by In situ Hybridization

1-23

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

**This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:**

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ **OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING:**

**This International Searching Authority found multiple inventions in this international application as follows:**

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

**Remark on Protest**

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.